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Analysis for Polychlorinated Dibenzo-p-Dioxins (PCDD) and Dibenzofurans (PCDF) in Human Adipose Tissue: Method Evaluation Study

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16. ABSTRACT

This report focuses on the evaluation of an HRGC/MS analytical method for determination of 2,3,7,8-substituted polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) in human adipose tissue. This method will be used for analysis of samples from EPA's National Human Adipose Tissue Survey (NHATS) as part of a collaborative effort between EPA's Office of Toxic Sub[illegible] and the Veterans Administration. The method was evaluated using aliquots of a bulk lipid matrix that was extracted from human adipose tissue. The results of the replicate analysis of spiked and unspiked homogenized human adipose tissue matrix demonstrate that the analytical method produces accurate and precise data for 17 specific 2,3,7,8-substituted PCDD and PCDF (tetra- through octachloro homologs) congeners. The endogenous or background levels of the PCDD and PCDF congeners in the homogenized adipose lipid matrix were estimated through regression analyses of measured versus spiked concentrations for each compound. This unspiked matrix will be used as a control sample with each batch of samples analyzed.

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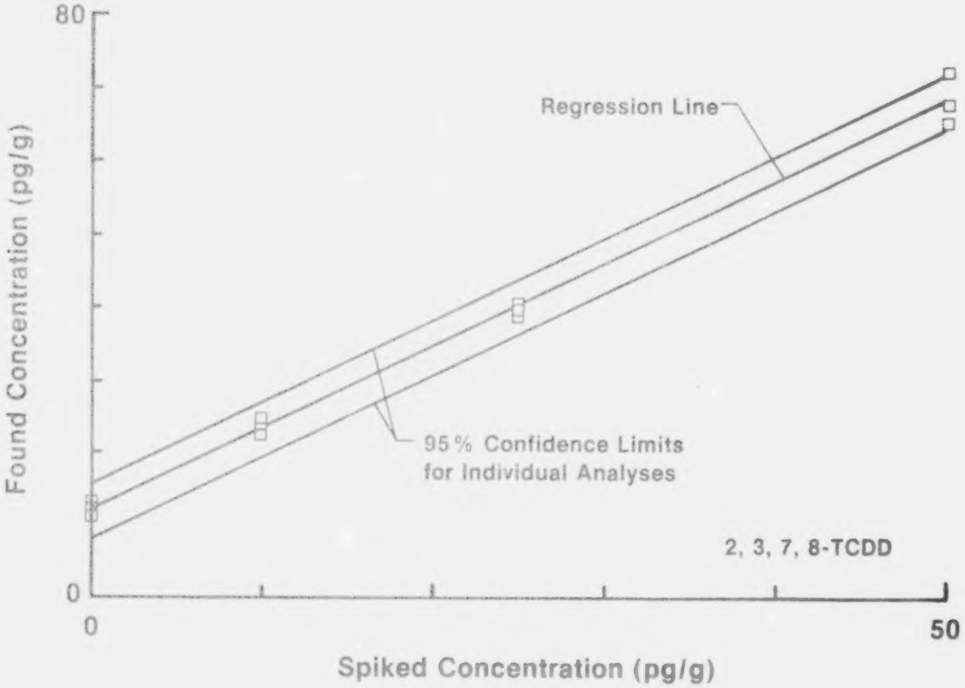

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by

John S. Stanley, Randy E. Ayling, Karin M. Bauer, Michael J. McGrath,
Thomas M. Sack, and Kelly R. Thornburg

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401 M Street, S.W.
Washington, DC 20460

Attn: Ms. Janet Remmers, Work Assignment Manager
Dr. Joseph J. Breen, Project Officer

1A

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PREFACE

This report provides a summary of the results from a method evaluation study for the determination of 2,3,7,8-substituted polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) in human adipose tissues at the parts-per-trillion (ppt) level. This method evaluation is an integral part of a collaborative program between the U.S. Environmental Protection Agency's Office of Toxic Substances and the Veterans Administration to determine if significant differences exist in the 2,3,7,8-substituted PCDD and/or PCDF levels in human adipose tissues for Vietnam veterans compared to the general adult male population. The study design will focus on specimens within EPA's National Human Adipose Tissue Survey (NHATS) repository. The method evaluation described in this report was necessary to establish method performance (accuracy and precision) before proceeding with actual sample analysis.

This method evaluation study was completed under EPA Contract Nos. 68-02-4252, Work Assignment 24 and 68-02-3938, Work Assignment 46, "Analysis for Dioxins and Furans in Human Adipose Tissue," Ms. Janet Remmers, Work Assignment Manager, and Dr. Joseph Breen, Project Officer.

MIDWEST RESEARCH INSTITUTE

John [illegible] for
Paul C. Constant
Program Manager

Approved:

Jack Balsinger
Jack Balsinger
Quality Assurance Coordinator

John E Going
John E. Going, Director
Chemical Sciences Department

October 30, 1986

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I. INTRODUCTION

The Environmental Protection Agency Office of Toxic Substances (EPA/OTS) and the Veterans Administration (VA) have established an interagency agreement to study the level of polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) in human adipose tissues. The occurrence and levels of PCDDs and PCDFs with chlorine substitution in the 2,3,7,8 positions (especially 2,3,7,8-TCDD) of the parent molecules are of primary interest.

As part of this interagency effort, it has been proposed to use selected adipose tissue samples that were collected for the Field Studies Branch (FSB) of EPA's Office of Toxic Substances (OTS) through the National Human Adipose Tissue Survey (NHATS) to determine exposure to PCDDs and PCDFs. The available adipose tissues include specimens obtained from young men whose age indicates that they could have served in Vietnam and could have been exposed to Agent Orange. The tissues were originally collected as part of a broadly based and statistical random sampling of the continental United States. The analysis of these tissues may provide information on the differences of exposure of the general adult male population and Vietnam veterans to the 2,3,7,8-substituted PCDDs and PCDFs.

The overall objectives of the proposed EPA/VA collaborative studies are:

1. Evaluate the reliability, accuracy, precision, and sensitivity of a proposed method for the determination of 2,3,7,8-substituted PCDDs and PCDFs (tetra- through octachloro homologs) in human adipose tissue at the parts-per-trillion (ppt) level.

2. Determine if these compounds can be detected in adipose tissues of the American male adult population; and

3. Determine if individuals with military service in Vietnam have significantly different levels of 2,3,7,8-substituted PCDDs and PCDFs (particularly 2,3,7,8-TCDD) than other American men.

As a prelude to this work assignment, MRI conducted an extensive literature review of applicable analytical methods and conducted a meeting with recognized experts in this field to identify critical aspects of analytical methodology.[1,2]

Based on the information gathered through the literature review and the meeting with the recognized experts, a special report was prepared for OTS proposing a framework for an analytical method for analysis of human adipose tissues.[3] Several studies have been completed since the issuance of that report which reflect the advances in analytical techniques for adipose tissue analysis.[4-16] The salient features of these methods have been combined into a single protocol for the routine analysis of tetra- through octachloro PCDDs and PCDFs at the low-parts-per-trillion level for the EPA/VA tissue study.

This report focuses on a method evaluation study that was conducted to achieve the first objective of the interagency agreement. Clarification of method performance is necessary before proceeding with the analysis of actual samples retrieved from the NHATS repository.

This report includes a summary of the method evaluation study results (Section II). Recommendations to be implemented before proceeding with the actual tissue samples from the NHATS repository are presented in Section III. A description of the actual experimental procedures is provided in Section IV. Results of sample analyses are summarized in Section V, and quality assurance/quality control (QA/QC) aspects of the study are detailed in Section VI. Pertinent references are listed in Section VII. Appendix A contains the detailed analytical protocol that will be followed for the analysis of the NHATS specimens designated in the study design to be provided by EPA/VA.

II. SUMMARY

The results of the replicate analysis of spiked and unspiked homogenized human adipose tissue matrix demonstrate that the analytical method produces accurate and precise data for 17 specific 2,3,7,8-substituted PCDD and PCDF (tetra- through octachloro homologs) compounds. Accuracy of the analytical method was demonstrated to range from 90 to 120% for the 17 2,3,7,8-substituted PCDD and PCDF compounds. Data are reported for three or four replicate analyses of samples spiked at three different concentration levels. The endogenous or background levels of the PCDD and PCDF congeners in the homogenized adipose lipid matrix were estimated through regression analyses of measured (found) versus spiked concentrations for each compound.

The analytical method is capable of providing quantitative data for tetra- through octachloro PCDD and PCDF congeners to concentration levels as low as 1 pg of the tetrachloro congeners per gram of adipose tissue. However, an interference was noted at m/z 304 which coeluted with 2,3,7,8-TCDF, resulting in a detection level of approximately 4 pg/g.

Average absolute recoveries of the internal quantitation standards ranged from 52% for $^{13}C_{12}$-TCDD up to 89% for $^{13}C_{12}$-OCDD. The agreement of the measured concentrations versus the spiked concentrations for each PCDD and PCDF congener demonstrates that the internal standard quantitation procedure provides an accurate measure of concentration which is independent of the absolute recovery.

Final concentration conditions were noted to have pronounced effect on the absolute recoveries of the lower chlorinated compounds, particularly 2,3,7,8-TCDD. Experiments with carbon-14 labeled 2,3,7,8-TCDD demonstrated that final concentration at temperatures of 55 to 60°C resulted in recoveries as low as 54% while the same procedure conducted at ambient conditions resulted in greater than 90% recovery.

Analysis of method and reagent blanks provided information on potential artifacts in the sample preparation scheme. Additional experiments were conducted with carbon-14 labeled PCDDs to evaluate the cleanup efficiency and recovery of PCDDs from chromatographic materials, particularly acidic alumina.

III. RECOMMENDATIONS

Some minor modifications have been made in the written protocol (Appendix A) that were not included in this phase of the method validation. These include:

- a cleanup procedure for activated acidic alumina prior to fractionation of sample extracts to remove artifacts; and
- final concentration of the sample extracts using nitrogen blowdown at room temperature rather than heating to 55-60°C.

The spiking solutions used to prepare the spiked quality control samples should be submitted for replicate (minimum of three/per spike level) HRGC/MS analysis to assist the interpretation of positive or negative bias in the accuracy of QC sample data.

The accuracy bounds should be extended to 50-130% from 50-115% as specified in the draft quality assurance program plan.

The method should include additional internal quantitation standards to pair with the HpCDF and OCDF congeners. Also, an additional internal recovery standard, possibly $^{13}C_{12}$-1,2,3,4,7,8-HxCDD, is required to provide better estimates of absolute method recovery. These additional compounds, if available, will be incorporated into the method before initiating sample analyses.

Analysis for 2,3,7,8-TCDF may require high resolution mass spectrometry to avoid interferences occurring at m/z 304. This will require modification of the HRGC/HRMS portion of the protocol to include the specific acquisition parameters for the characteristic ions of 2,3,7,8-TCDF.

IV. EXPERIMENTAL

A. Preparation of Homogenized Tissue

A bulk lipid sample was prepared from the extracts of human adipose tissue samples collected through the NHATS program. The adipose tissue samples have been stored in a deep freezer at approximately -10°C since collection. The homogenized tissue extract or bulk lipid was used in this method evaluation study for preparation of replicate samples spiked with varying levels of specific PCDD and PCDF isomers. This homogenized matrix will also be used for preparing control and spiked quality control samples for the actual NHATS sample analysis phase of the program.

A total of 2,465 g of adipose tissue was extracted, dried, and concentrated to yield 1,652 g (62% of original weight) of homogenized lipid. Specific procedures for preparing this matrix are described below.

The adipose tissue samples were thawed at room temperature for 1 to 2 h. Portions of the samples were added to a blender cup of a Waring® blender and covered with methylene chloride. The volume of methylene chloride was approximately equal to the sample volume (100 to 200 mL). This mixture was blended at high speed for approximately 10 min, and the contents were transferred to a 500-mL Erlenmeyer flask and further blended with a Tekmar® Tissumizer, also at high speed for 10 min. A powder funnel was plugged with a wad of glass wool (silanized, methylene chloride extracted) and filled with ~ 50 g of sodium sulfate (heated overnight to 600°C in a muffle furnace). The sodium sulfate was wetted with methylene chloride prior to elution of the sample extract. The dried effluents were refiltered in the same way using a fresh bed of sodium sulfate to remove particulate and residual water.

The samples were transferred to 1-L round bottom flasks, and the solvent was removed by rotary evaporation. The water bath on the rotary evaporator was kept at 60°C using a thermostatted heating element. Once the solvent appeared to have been removed (constant volume in flask, no visible condensation in condenser), the heating and evaporation process was continued for at least 2 h. The flask and contents were removed and stored in a refrigerator. The extracted lipid solidified upon refrigeration and was visually checked for homogeneity. No precipitates or phase separation was observed. The lipid residue was allowed to liquify at room temperature and was transferred to a 4-L glass bottle with a Teflon®-lined lid.

The lipid residue was brought to room temperature and heated just enough to allow the lipid to achieve an oily state prior to aliquotting portions for the method evaluation studies.

B. Analytical Standards

Analytical standards including native PCDD and PCDF congeners, stable isotope (carbon-13) labeled standards and radiolabeled (carbon-14) standards were purchased from Cambridge Isotope Laboratories, Woburn, Massachusetts, and Pathfinder Laboratories, St. Louis, Missouri. The 2,3,7,8-TCDD was received from the EPA Reference Materials Branch as a solution in isooctane. The other native PCDD and PCDF congeners were received as 1-mg neat standards. The stable and radiolabeled isotopes were received as solutions in n-nonane or isooctane and toluene, respectively. Table 1 provides a summary of the standards used for this study.

Stock solutions of the individual PCDD and PCDF congeners were prepared from the neat standards. The neat materials were weighed using a Cahn 27 electrobalance calibrated versus a 1-mg (Class M) standard. The neat compounds were transferred to glass vials and were dissolved in 2.0 to 3.0 mL of toluene (Burdick and Jackson, distilled in glass). Toluene was added to each standard using volumetric pipettes (Class A). The OCDD required dilution to 10.0 mL using a 50:50 mixture of toluene and anisole.

A working solution consisting of the 17 native PCDD and PCDF congeners was prepared in toluene at a concentration of 2 µg/mL for the TCDD, TCDF, PeCDD, and PeCDF congeners, 5 µg/mL for the HxCDD, HxCDF, HpCDD, and HpCDF congeners, and 10 µg/mL for the OCDD and OCDF. The working solution was used to prepare both the lipid matrix spiking solution and the calibration standards.

The stable isotope labeled internal standards were obtained as solutions in n-nonane or isooctane at 50 µg/mL concentration with the exception of the $^{13}C_{12}$-OCDD, which was provided at 10 µg/mL. Separate working solutions containing mixtures of the carbon-13 labeled PCDDs and PCDFs were prepared in isooctane for use in the calibration standards and the sample spiking solutions.

The carbon-14 radiolabeled PCDDs were used for preliminary method evaluation studies. The specific activity of the ^{14}C-2,3,7,8-TCDD (117.56 mCi/mmole) was high enough to allow recovery studies at spike levels equivalent to 10 pg/g for a 10-g sample.

1. Calibration Standards

Eight concentration calibration standards containing the 17 native and the 9 carbon-13 labeled internal standards were prepared for determining the consistency of response factors for the native PCDDs and PCDFs versus the corresponding carbon-13 congeners. Table 2 presents a summary of the calibration standards prepared for the method calibration study. The solution concentrations (pg/µL) can also be considered as equivalent to residue levels in picograms per gram of adipose. For example, a 1 pg/µL concentration standard corresponds to a tissue concentration of 1 pg of PCDD or PCDF congener per gram of adipose assuming a 10-g sample is available for analysis.

2. Spiking Solutions

a. Native PCDD and PCDF

A solution containing the 2,3,7,8-substituted PCDD and PCDF congeners was prepared in isooctane for spiking the homogenized lipid materials for the method evaluation study. Table 3 specifies the levels of each of the native PCDD and PCDF congeners present in this solution.

Table 1. Analytical Standards Available for the Method Evaluation Studies

Compound	Source	Lot/Code
Native		
2,3,7,8-TCDD	EPA QA Reference Materials Branch	20603
2,3,7,8-TCDF	Cambridge Isotope Laboratories	AWN 1203-74/EF-903C
1,2,3,7,8-PeCDD	Cambridge Isotope Laboratories	MLB-706-53/ED-950C
1,2,3,7,8-PeCDF	Cambridge Isotope Laboratories	AWN-729-21/EF-953C
2,3,4,7,8-PeCDF	Cambridge Isotope Laboratories	AWN-729-45/EF-956C
1,2,3,4,7,8-HxCDD	Cambridge Isotope Laboratories	830244/ED-961C
1,2,3,6,7,8-HxCDD	Cambridge Isotope Laboratories	MLB-706-47/ED-960C
1,2,3,7,8,9-HxCDD	Cambridge Isotope Laboratories	MLB-706-73/ED-969C
1,2,3,4,7,8-HxCDF	Cambridge Isotope Laboratories	AWN-729-20/EF-964C
1,2,3,6,7,8-HxCDF	Cambridge Isotope Laboratories	MB 13106-7/EF-962-C
1,2,3,7,8,9-HxCDF	Cambridge Isotope Laboratories	MB 13106-47/EF-967-C
2,3,4,6,7,8-HxCDF	Cambridge Isotope Laboratories	MB 13106-3/EF-968-C
1,2,3,4,6,7,8-HpCDD	Cambridge Isotope Laboratories	MLB-706-21/ED-971C
1,2,3,4,6,7,8-HpCDF	Cambridge Isotope Laboratories	AWN-729-22/EF-973C
1,2,3,4,7,8,9-HpCDF	Cambridge Isotope Laboratories	MB-13-106-77/EF-975C
OCDD	Cambridge Isotope Laboratories	8465-F-982-C/EF-982C
OCDF	Cambridge Isotope Laboratories	F2832/ED-980C
$^{13}C_{12}$-Internal standards		
2,3,7,8-TCDD	Cambridge Isotope Laboratories	R00208/ED-900
2,3,7,8-TCDF	Cambridge Isotope Laboratories	R00236/EF-904
1,2,3,7,8-PeCDD	Cambridge Isotope Laboratories	R00241/ED-955
1,2,3,7,8-PeCDF	Cambridge Isotope Laboratories	R00221/EF-952
1,2,3,6,7,8-HxCDD	Cambridge Isotope Laboratories	R00249/ED-966C
1,2,3,4,7,8-HxCDF	Cambridge Isotope Laboratories	R00234/EF-963C
1,2,3,4,6,7,8-HpCDD	Cambridge Isotope Laboratories	R00248/ED-972
OCDD	Cambridge Isotope Laboratories	R00263/ED-981
^{37}Cl-Internal standard		
$^{37}Cl_4$-1,2,3,4,6,7,8-HpCDD	KOR Isotopes	580012/SSY-4-32
$^{14}C_{12}$-Radiolabeled standards		
2,3,7,8-TCDD	Pathfinder Laboratories	S.A.[a] = 117.56 mCi/mmole
1,2,3,4,7,8-HxCDD	Pathfinder Laboratories	S.A. = 24.16 mCi/mmole
OCDD	Pathfinder Laboratories	S.A. = 20.50 mCi/mmole

---

[a] S.A. = specific activity.

Table 2. Concentration Calibration Solutions[a]

Compound	Concentration in calibration solutions in pg/μL							
	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8
Native								
2,3,7,8-TCDD	200	100	50	25	10	5	2.5	1
2,3,7,8-TCDF	200	100	50	25	10	5	2.5	1
1,2,3,7,8-PeCDD	200	100	50	25	10	5	2.5	1
1,2,3,7,8-PeCDF	200	100	50	25	10	5	2.5	1
2,3,4,7,8-PeCDF	200	100	50	25	10	5	2.5	1
1,2,3,4,7,8-HxCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,6,7,8-HxCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,7,8,9-HxCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,7,8-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,6,7,8-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,7,8,9-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
2,3,4,6,7,8-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,6,7,8-HpCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,6,7,8-HpCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,7,8,9-HpCDF	500	250	125	62.5	25	12.5	6.25	2.5
OCDD	1,000	500	250	125	50	25	12.5	5
OCDF	1,000	500	250	125	50	25	12.5	5
Internal quantitation standards								
$^{13}C_{12}$-2,3,7,8-TCDD	50	50	50	50	50	50	50	50
$^{13}C_{12}$-2,3,7,8-TCDF	50	50	50	50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8-PeCDD	50	50	50	50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8-PeCDF	50	50	50	50	50	50	50	50
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	125	125	125	125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	125	125	125	125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	125	125	125	125	125	125	125	125
$^{13}C_{12}$-OCDD	250	250	250	250	250	250	250	250
Internal recovery standard								
$^{13}C_{12}$-1,2,3,4-TCDD	50	50	50	50	50	50	50	50

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[a] Prepared in tridecane.

Table 3. Native PCDD and PCDF Spiking Solution[a]

Compound	Concentration (pg/μL)
2,3,7,8-TCDD	5
2,3,7,8-TCDF	5
1,2,3,7,8-PeCDD	5
1,2,3,7,8-PeCDF	5
2,3,4,7,8-PeCDF	5
1,2,3,4,7,8-HxCDD	12.5
1,2,3,6,7,8-HxCDD	12.5
1,2,3,7,8,9-HxCDD	12.5
1,2,3,4,7,8-HxCDF	12.5
1,2,3,6,7,8-HxCDF	12.5
1,2,3,7,8,9-HxCDF	12.5
2,3,4,6,7,8-HxCDF	12.5
1,2,3,4,6,7,8-HpCDD	12.5
1,2,3,4,6,7,8-HpCDF	12.5
1,2,3,4,7,8,9-HpCDF	12.5
OCDD	25
OCDF	25

[a] Prepared in isooctane.

b. Internal Standards

Two different internal standard spiking solutions were prepared for quantitation of native PCDD and PCDF congeners. The compositions of each of the spiking solutions are presented in Table 4. The internal quantitation standards were spiked into the lipid aliquots prior to any cleanup procedures and hence were carried throughout the method exactly as the corresponding native congeners. The internal recovery standard was added in 10 µL of a keeper solution (tridecane) during final extract concentration prior to analysis. The recovery standard was used to measure the absolute method recoveries of the internal quantitation standards.

C. Analytical Procedure

The homogenized human adipose lipid matrix was allowed to come to room temperature and then warmed in a water bath until the matrix changed to an oily state. Approximately 10.0 g of the oily material was transferred by pipette to preweighed glass vials, and the actual weight of the lipid was determined to the nearest 0.01 g by difference using an analytical balance. Four 10.00-g aliquots were spiked with 20 µL of the native spiking solution presented in Table 3, another four aliquots were spiked with 50 µL of the same solution, and three additional aliquots were spiked with 100 µL of native PCDD and PCDF solution. These spikes were equivalent to concentrations ranging from 10, 25, and 50 pg/g in the lipid matrix for the tetra- and pentachloro PCDD and PCDF congeners up to 50, 125, and 250 pg/g for the OCDD and OCDF for the low, medium, and high level spikes.

In addition to the spiked samples, three aliquots of the lipid material were transferred for determining the endogenous levels of each of the PCDD and PCDF congeners in the control matrix.

Each of the sample aliquots was fortified with 100 µL of the internal quantitation standard spiking solution (Table 4). The spiked samples were each quantitatively transferred to 500-mL Erlenmeyer flasks using hexane.

The residues were diluted with a total of 200 mL of hexane, and 100 g of sulfuric acid (H_2SO_4) modified silica gel (40% w/w) was added to each solution with stirring. The mixtures were stirred for approximately 2 h, and the supernatants were decanted and filtered through filter funnels packed with anhydrous sodium sulfate (Na_2SO_4). The H_2SO_4 modified silica adsorbents were washed with at least two additional aliquots of hexane and dried by elution through Na_2SO_4.

The combined hexane extracts for each sample were eluted through a column consisting of the 40% H_2SO_4 modified silica gel (4.0 g) and silica gel (1.0 g). The eluates were concentrated to approximately 15 mL and added to columns of acidic alumina (Bio-Rad, AG-4, 6.0 g). The acidic alumina columns were eluted first with 20 mL of hexane, which was collected but not analyzed, followed by elution with 30 mL of 20% methylene chloride in hexane. The PCDDs and PCDFs were eluted from the acidic alumina using the 20% methylene chloride in hexane. The PCDDs and PCDFs in the eluates were isolated from other chlorinated planar aromatics using columns (5-mL disposable pipettes containing 500 mg of 18% Carbopak C and Celite-545). The Carbopak C/Celite

Table 4. Internal Standard Spiking Solutions

Compound	Concentration (pg/µL)
Internal quantitation standard[a]	
$^{13}C_{12}$-2,3,7,8-TCDD	5
$^{13}C_{12}$-2,3,7,8-TCDF	5
$^{13}C_{12}$-1,2,3,7,8-PeCDD	5
$^{13}C_{12}$-1,2,3,7,8-PeCDF	5
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	12.5
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	12.5
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	12.5
$^{13}C_{12}$-OCDD	25
Internal recovery standard[b]	
$^{13}C_{12}$-1,2,3,4-TCDD	50

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[a] Solution prepared in isooctane.
[b] Solution prepared in tridecane.

columns were pre-eluted with 2 mL of toluene, 1 mL of 75:20:5 methylene chloride/methanol/benzene, 1 mL of 1:1 methylene chloride/cyclohexane, and 2 mL of hexane. The sample extracts (30 mL) were added to the columns, which were eluted with 2 mL of hexane, 1 mL of 1:1 methylene chloride/cyclohexane, and 1 mL of the 75:20:5 methylene chloride/methanol/benzene. These eluents were collected and combined but were not analyzed. The Carbopak C/Celite columns were turned upside down, and the PCDDs and PCDFs were eluted with 20 mL of toluene. The toluene was concentrated to less than 1 mL using flowing nitrogen and a heated water bath (55-60°C) and transferred to 1.0-mL conical vials using a solution of 1% toluene in methylene chloride. Tridecane (10 µL) containing 500 pg of the internal recovery standard $^{13}C_{12}$-1,2,3,4-TCDD was added as a keeper when the solution had concentrated to approximately 200 µL. The extracts were concentrated to final volume using nitrogen and the heated water bath.

D. HRGC/MS Analysis

The analyses of the spiked and unspiked lipid samples were completed using a Kratos MS50TC double-focusing magnetic sector mass spectrometer. The determination for the tetra- through octachloro homologs was achieved in a single analysis using the conditions described in Table 5. Table 6 provides the characteristic ions monitored for each PCDD and PCDF homolog. As noted from Table 6, the analysis requires five different parameter descriptions that were switched automatically during the course of the analysis. Parameters monitored included two characteristic molecular ions for each PCDD and PCDF homolog and the corresponding carbon-13 labeled internal standard. In addition, a fragment ion of perfluorokerosene (PFK), m/z 380.976, was monitored throughout each analysis to ensure that proper mass calibration was maintained. The parameter descriptors also included an ion characteristic of specific homologs of chlorinated diphenyl ethers to demonstrate that responses meeting the qualitative criteria for specific PCDF congeners were not due to these potential interferences.

Triplicate analyses of six of the eight calibration solutions (Table 2) were completed, and the variability in relative response factors across this range was calculated. The analyst was required to demonstrate on a daily basis that the relative response factors (RRF) were in agreement within ± 20% of the established averages for 2,3,7,8-TCDD and 2,3,7,8-TCDF and within ± 30% of the average RRF values for the other congeners. The equation used to calculate the relative response factors for each PCDD and PCDF congener are discussed later in this report (Section E - Data Interpretation, 2 - Quantitation, p. 17). The analyst was also required to determine column performance by analyzing a mixture of TCDD isomers before proceeding with sample analysis. Table 7 gives an example of the typical daily sequence for PCDD/PCDF analysis.

Table 5. HRGC/LRMS Operating Conditions for PCDD/PCDF Analysis

Mass spectrometer	
Accelerating voltage:	8,000 V
Trap current:	500 μA
Electron energy:	70 eV
Electron multiplier voltage:	-1,800 V
Source temperature:	280°C
Resolution:	≧ 3,000 (10% valley definition)
Overall SIM cycle time:	1 s
Gas chromatograph	
Column coating:	DB-5
Film thickness:	0.25 μm
Column dimensions:	60 m x 0.25 mm ID
He linear velocity:	∿ 25 cm/sec
He head pressure:	1.75 kg/cm^2 (25 psi)
Injection type:	Splitless, 45 s
Split flow:	30 mL/min
Purge flow:	6 mL/min
Injector temperature:	270°C
Interface temperature:	300°C
Injection size:	1-2 μL
Initial temperature:	200°C
Initial time:	2 min
Temperature program:	200°C to 330°C at 5°C/min

Table 6. Ions Monitored for HRGC/MS Analysis of PCDD/PCDF

Descriptor	ID	Mass	Nominal dwell time (sec)
A1	TCDF	303.902	0.090
		305.899	0.090
	$^{13}C_{12}$-TCDF	315.942	0.090
		317.939	0.090
	TCDD	319.897	0.090
		321.894	0.090
	$^{13}C_{12}$-TCDD	331.937	0.090
		333.934	0.090
	HxCDPE	373.840	0.090
	PFK (lock mass)	380.976	0.090
A2	TCDF	303.902	0.045
		305.899	0.045
	TCDD	319.897	0.045
		321.894	0.045
	PeCDF	337.863	0.045
		339.860	0.045
	$^{13}C_{12}$-PeCDF	349.903	0.045
		351.900	0.045
	PeCDD	353.858	0.045
		355.855	0.045
	$^{13}C_{12}$-PeCDD	365.898	0.045
		367.895	0.045
	PFK (lock mass)	380.976	0.035
	HpCDPE	407.801	0.035
A3	HxCDF	373.821	0.080
		375.818	0.080
	PFK (lock mass)	380.976	0.080
	$^{13}C_{12}$-HxCDF	385.861	0.080
		387.859	0.080
	HxCDD	389.816	0.080
		391.813	0.080
	$^{13}C_{12}$-HxCDD	401.856	0.080
		403.853	0.080
	OCDPE	443.759	0.080

Table 6 (continued)

Descriptor	ID	Mass	Nominal dwell time (sec)
A4	PFK (lock mass)	380.976	0.040
	HxCDD	389.816	0.040
		391.813	0.040
	HpCDF	407.782	0.040
		409.779	0.040
	$^{13}C_{12}$-HpCDF	419.822	0.040
		421.819	0.040
	HpCDD	423.777	0.040
		425.774	0.040
	$^{13}C_{12}$-HpCDD	435.817	0.040
		437.814	0.040
	$^{37}Cl_4$-HpCDD	429.768	0.040
		431.765	0.040
	NCDPE	477.720	0.040
A5	PFK (lock mass)	380.976	0.060
	OCDF	441.743	0.070
		443.740	0.070
	$^{13}C_{12}$-OCDF	453.784	0.070
		455.781	0.070
	OCDD	457.738	0.070
		459.735	0.070
	$^{13}C_{12}$-OCDD	469.779	0.070
		471.776	0.070
	DCDPE	511.681	0.060

Table 7. Typical Daily Sequence for PCDD/PCDF Analysis

1. Tune and calibrate mass scale versus perfluorokerosene (PFK).
2. Determine column performance by injecting the TCDD isomer mixture.
3. Inject concentration calibration solution 2.5 to 12.5 pg/μL (CS-7) solution.
4. Inject blank (tridecane).
5. Inject samples 1 through "n".

6 Inject concentration calibration solution 2.5 to 12.5 pg/μL (CS-7) solution.

E. Data Interpretation

1. Qualitative

The HRGC/MS elution profiles of the tetra- through octachloro PCDD and PCDF homologs were established through the analysis of environmental sample extract (fly ash from a municipal waste incinerator). The characteristic ions for each homolog were plotted within the retention window established using this mixture. The criteria for identification of a response as a PCDD or PCDF were the coincidental response of the characteristic ions monitored within the established retention window, and within ± 20% of the theoretical ion ratio. Table 8 presents the range of ion ratios used for the qualitative criteria for the specific PCDD and PCDF homologs and internal standards.

2. Quantitation

Quantitation of the specific PCDD and PCDF congeners was achieved using the respective internal quantitation standards. For example, TCDD was quantitated versus the $^{13}C_{12}$-2,3,7,8-TCDD; PeCDD versus the $^{13}C_{12}$-1,2,3,7,8-PeCDD, etc. The HpCDF and OCDF responses were quantitated versus the carbon-13 labeled hepta- and octachlorodibenzo-p-dioxin internal standards since the corresponding dibenzofuran internal standards were not available for this study. The absolute recovery of the internal quantitation standards was achieved using $^{13}C_{12}$-1,2,3,4-TCDD. A second internal recovery standard, $^{37}Cl_4$-1,2,3,4,6,7,8-HpCDD, was evaluated but was not used for recovery measurements due to interference arising from the corresponding native HpCDD.

Relative response factors (RRF) were calculated for each of the native PCDD and PCDF compounds listed in Table 2. The RRF values were calculated as shown in Equation 1.

$$RRF = \frac{A_{STD} \times C_{IS}}{A_{IS} \times C_{STD}} \qquad \text{Eq. 1}$$

where A_{STD} = the sum of the area responses for the two characteristic ions of the standard compound;

A_{IS} = the sum of the area responses for the two characteristic ions of the corresponding internal quantitation standard;

C_{IS} = concentration (pg/µL) of the internal quantitation standard; and

C_{STD} = concentration (pg/µL) of the standard compound.

The relative response factors for the internal quantitation standards (RRF_{IS}) were calculated as shown in Eq. 2.

Table 8. Ion Ratios for HRGC/MS Analysis of PCDD/PCDF

Compound	Ions monitored	Theoretical ratio	Acceptable range[a]
TCDF	304/306	0.76	0.61 - 0.91
$^{13}C_{12}$-TCDF	316/318	0.76	0.61 - 0.91
TCDD	320/322	0.76	0.61 - 0.91
$^{13}C_{12}$-TCDD	332/334	0.76	0.61 - 0.91
PCDF	338/340	0.61	0.49 - 0.73
$^{13}C_{12}$-PeCDF	350/352	0.61	0.49 - 0.73
PeCDD	354/356	0.61	0.49 - 0.73
$^{13}C_{12}$-PeCDD	366/368	0.61	0.49 - 0.73
HxCDF	374/376	1.22	0.98 - 1.46
$^{13}C_{12}$-HxCDF	386/388	1.22	0.98 - 1.46
HxCDD	390/392	1.22	0.98 - 1.46
$^{13}C_{12}$-HxCDD	402/404	1.22	0.98 - 1.46
HpCDF	408/410	1.02	0.82 - 1.22
$^{13}C_{12}$-HpCDF	420/422	1.02	0.82 - 1.22
HpCDD	424/426	1.02	0.82 - 1.22
$^{13}C_{12}$-HpCDD	436/438	1.02	0.82 - 1.22
OCDF	442/444	0.87	0.70 - 1.04
$^{13}C_{12}$-OCDF	454/456	0.87	0.70 - 1.04
OCDD	458/460	0.87	0.70 - 1.04
$^{13}C_{12}$-OCDD	470/472	0.87	0.70 - 1.04

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[a] Acceptable range is ± 20% of the theoretical value.

$$RRF_{IS} = \frac{A_{IS} \times C_{RS}}{A_{RS} \times C_{IS}} \qquad \text{Eq. 2}$$

where A_{IS} and C_{IS} are defined as in Equation 1 and

C_{RS} = concentration (pg/µL) of the internal recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD, and

A_{RS} = the sum of the area responses for the two characteristic ions (m/z 332 and 334) corresponding to the internal recovery standard.

A calibration curve was established using six concentration levels of standards; for example, the calibration curve for 2,3,7,8-TCDD was initially established with standards at concentrations of 1, 2.5, 5, 10, 50, and 100 pg/µL. The 2.5 pg/µL standard was analyzed daily to verify response factors and instrument sensitivity. The RRF values for each of the internal quantitation standards were calculated versus the internal recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD, using Equation 2.

The concentration of a PCDD or PCDF congener in a composite sample was calculated as shown in Equation 3.

$$C_{WT} = \frac{A_{sample} \times Q_{IS}}{A_{IS} \times RRF \times Wt} \qquad \text{Eq. 3}$$

where C_{WT} = wet tissue concentration of the PCDD or PCDF congener in each tissue (pg/g);

A_{sample} = sum of the area responses for the two characteristic ions of the PCDD or PCDF congener;

A_{IS} = sum of the area responses for the two characteristic ions of the respective internal quantitation standard;

Q_{IS} = amount of the internal quantitation standard added to the sample (500 pg of $^{13}C_{12}$-TCDD, $^{13}C_{12}$-TCDF, $^{13}C_{12}$-PeCDD, and $^{13}C_{12}$-PeCDF; 1,250 pg of $^{13}C_{12}$-HxCDD, $^{13}C_{12}$-HxCDF, and $^{13}C_{12}$-HpCDD; or 2,500 pg of $^{13}C_{12}$-OCDD);

RRF = the relative response factor for the PCDD or PCDF congener from Equation 1; and

Wt = mass of the sample (grams).

The absolute recovery of the internal quantitation standard was calculated using Equation 4.

$$\text{Recovery (\%)} = \frac{A_{IS} \times Q_{RS}}{A_{RS} \times RRF_{IS} \times Q_{IS}} \times 100 \qquad \text{Eq. 4}$$

where A_{RS} = sum of the area responses for the two characteristic ions of the internal recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD;

Q_{RS} = amount of the internal recovery standard ($^{13}C_{12}$-1,2,3,4-TCDD) added to the final extract (500 pg); and

RRF_{IS} = response factor of the internal quantitation standard relative to the internal recovery standard. These values are calculated as defined in Equation 2. The RRF_{IS} values were all calculated versus $^{13}C_{12}$-1,2,3,4-TCDD.

All data were qualified to reflect that the response for a particular compound was a positive quantifiable parameter, present as a trace value only, or was not detected. Positive quantifiable values were identified for responses greater than 10 times background signal to noise. Trace (TR) values were assigned to responses which were in the range of 2.5 to 10 times background signal to noise. A value of not detected (ND) was used to reflect that a response was not detected at greater than 2.5 times signal to noise. A limit of detection (LOD) was calculated for all trace and not detected values using the peak height response of the respective internal standard and the average measured signal to noise for the characteristic ions of the PCDDs and PCDFs.

F. Quality Assurance/Quality Control (QA/QC)

The QA/QC procedures included analysis of multipoint calibration concentration standards to establish relative response factor (RRF) curves for each of the 17 native PCDD and PCDF congeners. Triplicate analyses of 6 concentration calculation standards (Table 2; CS2, CS3, CS5, CS6, CS7, and CS8) were determined to vary by less than ± 20% for TCDD and TCDF and ± 30% for all other PCDD and PCDF congeners. The mean RRF values were also determined to vary by less than this criteria over the entire calibration range. The mean RRF ($\overline{RRF}$) values and instrument sensitivity were verified daily by bracketing the sample analyses with an injection of a standard that ranged from 2.5 pg/µL for 2,3,7,8-TCDD and 2,3,7,8-TCDF up to 12.5 pg/µL for OCDD and OCDF. The criterion for continuing with the sample analysis was agreement of the measured RRF value with the mean RRF within ± 20% for 2,3,7,8-TCDD and TCDF and ± 30% for all other PCDD and PCDF congeners.

Other activities included the analysis of laboratory method blanks and reagent blanks and measurement of the absolute recoveries of the internal quantitation standards. Laboratory method blanks were samples that were handled exactly as an adipose sample except no lipid matrix was used.

G. Preliminary Method Studies

Prior to analysis of the homogenized human adipose lipid matrix by HRGC/MS, several experiments were conducted to confirm that the sample preparation scheme was feasible.

1. Gravimetric Studies

The first concern was the efficient removal of up to 10 g of lipid matrix from extracted adipose tissue. A series of experiments was conducted with 10-g lipid aliquots to demonstrate removal of lipid using the H_2SO_4-SiO_2 slurry technique. Initially, 50 g of the acid modified silica was added to the lipid extract in 100 mL of hexane. The acid modified silica was noted to turn dark brown immediately on contact with the lipid solution. The hexane was recovered and the adsorbent was extracted with additional hexane. The extracts were combined and concentrated to 5 mL with Kuderna-Danish evaporators. The extract was eluted through a column of 4.0 g of acid modified silica and 1.0 g of silica with 45 mL of hexane. The acid modified silica was noted to be highly discolored throughout, and the extract required a second slurry treatment of the eluent with an additional 50 g of acid modified silica gel. The adsorbent from the second slurry procedure was noted to discolor significantly, indicating that lipid materials had not been efficiently removed from the first step of the procedure. The hexane supernatant from the second slurry cleanup was reduced in volume and taken to dryness in a preweighed glass vial. The final residue was measured at approximately 10 mg for duplicate samples, which translates into a removal efficiency of 99.9% based on the initial 10-g aliquot.

This lipid cleanup procedure was modified such that 100 g of acid modified silica gel was used in the initial slurry cleanup, followed by elution of the resulting extract through a column containing 4.0 g of acid modified silica and 1.0 g of silica gel. The lipid removal efficiency of duplicate samples through the cleanup procedure was determined to average 99.8% (20 to 30 mg of the initial 10-g lipid remained after cleanup). The column cleanup step in this procedure did not exhibit any significant color change. Thus this step of the procedure was incorporated into the method as a check of the efficiency of lipid removal to prevent overloading of the acidic alumina fractionation column.

2. Carbon-14 Recovery Studies

The carbon-14 radiolabeled PCDD standards listed in Table 1 were used to estimate overall method recoveries for the tetra- through octachloro homologs prior to proceeding with the HRGC/MS evaluation. Triplicate analyses (10-g aliquots of lipid materials) were conducted with each of the three radiolabeled standards. The first experiment addressed the recovery of the compounds from bulk lipid cleanup. Triplicate analyses using 10-g aliquots were completed for the three compounds at the following concentrations: ^{14}C-2,3,7,8-TCDD, 10 pg/g; ^{14}C-1,2,3,4,7,8-HxCDD, 100 pg/g; and ^{14}C-OCDD, 250 pg/g. The results of these analyses indicated that all compounds were recovered in the range of approximately 70 to 80%. Following this experiment, the total sample preparation procedure described earlier in this report was evaluated using triplicate analysis of 10-g lipid samples. Table 9 provides a summary of the results from this study. These data indicate that overall method recovery is limited by the initial bulk lipid removal procedure. This assumption is based on the similar recoveries of the carbon-14 labeled compounds noted for evaluation of the bulk lipid removal step as compared to the total sample preparation scheme.

Table 9. Summary of the Results of the Sample Preparation Method Evaluation Using Carbon-14 PCDDs

Analytes	Spike levels (pg/g)	Total method recovery (%)[a]	Bulk lipid removal recovery[b]
^{14}C-2,3,7,8-TCDD	10	68	75
^{14}C-1,2,3,4,7,8-HxCDD	100	79	66
^{14}C-OCDD	250	82	76

[a] Average value for triplicate analyses taken through the total sample preparation scheme. Precision of measurements varied by less than ± 10% (relative standard deviation).

[b] Average value for triplicate analyses taken through bulk lipid cleanup only. Precision of measurement varied by less than ± 6% (relative standard deviation).

V. RESULTS

A. Analytical Results

The analytical results for the quantitation of the 17 target PCDD and PCDF 2,3,7,8-substituted congeners in the spiked and unspiked homogenized human adipose lipid samples are presented in Tables 10 to 15. These data demonstrate that 13 of the 17 congeners were definitely detected in the unspiked lipid matrix. Although 2,3,7,8-TCDF is reported as not detected, responses for the characteristic ions (m/z 304 and 306) greater than 10 times signal to noise were noted to be coincident with the internal standard, $^{13}C_{12}$-2,3,7,8-TCDF. The ratio of the responses (m/z 304/306) in each of the triplicate analyses of the unspiked matrix were well outside the acceptable ratio of 0.90 to 0.61 established in Table 8. Figure 1 provides a comparison of the HRGC/MS-SIM responses noted for the unpsiked human adipose lipid matrix as compared to a concentration calibration standard. Figures 2 through 6 provide examples of the individual PCDD and PCDF responses observed for the unspiked lipid samples as compared to fortified matrices.

In general, the precision of the replicate measurements at each spike level is good (relative standard deviations typically less than 10%) for PCDD and PCDF congeners that were detected with responses greater than 10 times signal to noise. The precision of the measurements for the unspiked matrices for 1,2,3,7,8-PeCDF (Table 11), 1,2,3,7,8,9-HxCDF (Table 12), and OCDF (Table 15) ranges from 21.6% to 43.1% as a result of little or no response at the specified retention windows.

B. Statistical Analysis

The regression results for each of the 17 specific PCDD and PCDF congeners are plotted separately in Figures 7 to 23. These plots provide the results for the individual sample analyses, a line defining the results of a least squares regression analysis, and boundaries that depict the confidence limits for the range of spiked concentrations. The regression lines were obtained by the method of least squares using the sample measurements at the three spiking levels and the unspiked level.

Two types of upper and lower 95% confidence limits or bounds were calculated for the least square regressions of measured (found) concentrations versus spiked levels. The first set of confidence limits (defined by the inner pair of curves closest to the regression line) is the 95% confidence bounds for the regression line. These bounds are interpreted as follows: The true regression line (as would be determined if the experiment were repeated a countless number of times at the same spiked levels) lies within these confidence limits unless the analytical results are sufficiently unusual to be among those expected to occur less than 5% of the time.

The second set of confidence bounds, depicted by the outer pair of lines, constitutes the 95% confidence limits for the result of a single analysis at a particular spiking level. The interpretation is as follows: the result (reported value) of a single analysis of a sample spiked at a given level can be predicted to fall between these 95% confidence bounds unless the analytical result is among those sufficiently unusual to be expected less than 5% of the time.

Table 10. Spiked Versus Measured Concentrations of 2,3,7,8-TCDF and 2,3,7,8-TCDD in Homogenized Human Adipose Lipid Samples

2,3,7,8-TCDF spike level (pg/g)	2,3,7,8-TCDF concentration (pg/g)	$^{13}C_{12}$-TCDF absolute recovery (%)	2,3,7,8-TCDD spike level (pg/g)	2,3,7,8-TCDD concentration (pg/g)	$^{13}C_{12}$-TCDD absolute recovery (%)
0	ND (4.1)[a]	59	0	10.7	53
0	ND (4.1)[a]	63	0	11.4	52
0	ND (4.0)[a]	59	0	13.1	46
Mean	ND (4.1)	60.3		11.7	50.3
STD	0.1	2.3		1.2	3.8
RSD (%)	1.8	3.8		10.5	7.5
10	14.3	61	10	23.4	53
10	14.8	67	10	22.8	53
10	13.6	71	10	24.7	53
10	13.8	78	10	22.5	62
Mean	14.1	69.3		23.3	55.3
STD	0.5	7.1		1.0	4.5
RSD (%)	3.9	10.3		4.1	8.1
25	30.8	59	25	40.8	48
25	30.8	75	25	40.6	53
25	30.7	62	25	40.3	51
25	28.7	72	25	38.3	60
Mean	30.2	67.0		40.0	53.0
STD	1.1	7.7		1.2	5.1
RSD (%)	3.5	11.5		2.9	9.6
50	57.7	64	50	65.8	55
50	59.4	48	50	72.1	43
50	55.8	58	50	67.6	48
Mean	57.6	56.7		68.5	48.7
STD	1.8	8.1		3.3	6.0
RSD (%)	3.1	14.3		4.8	12.4

[a] ND = not detected. Value in parentheses is the estimated limit of detection. A response of greater than 10 times signal-to-noise was noted for both characteristic ions (m/z 304 and 306) at the appropriate retention time for 2,3,7,8-TCDF. However, the ion ratio was considerably greater than the acceptable range of 0.61 to 0.90.

Table 11. Spiked Versus Measured Concentrations of 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, and 1,2,3,7,8-PeCDD in Homogenized Human Adipose Tissue Samples

	1,2,3,7,8-PeCDF spike level (pg/g)	1,2,3,7,8-PeCDF concentration (pg/g)	2,3,4,7,8-PeCDF spike level (pg/g)	2,3,4,7,8-PeCDF concentration (pg/g)	$^{13}C_{12}$-PeCDF absolute recovery (%)	1,2,3,7,8-PeCDD spike level (pg/g)	1,2,3,7,8-PeCDD concentration (pg/g)	$^{13}C_{12}$-PeCDD absolute recovery (%)
	0	ND (1.1)[a]	0	20.8	75	0	20.2	51
	0	ND (0.8)	0	21.6	76	0	19.9	54
	0	ND (0.8)	0	19.0	80	0	18.1	57
Mean		ND (0.9)		20.5	77.0		19.4	54.0
STD		0.2		1.3	2.6		1.1	3.0
RSD (%)		21.6		6.5	3.4		5.7	5.6
	10	12.2	10	27.6	90	10	32.1	60
	10	11.5	10	37.0	63	10	37.6	57
	10	11.9	10	28.7	84	10	30.8	55
	10	11.4	10	36.3	75	10	30.4	62
Mean		11.8		32.4	78.0		30.2	58.5
STD		0.4		4.9	11.7		1.9	3.1
RSD (%)		3.1		15.2	15.1		6.3	5.3
	25	29.2	25	54.9	62	25	48.0	55
	25	30.1	25	48.5	81	25	46.1	60
	25	28.5	25	41.7	87	25	49.3	57
	25	25.5	25	48.2	84	25	43.5	64
Mean		28.3		48.4	78.5		46.7	59.0
STD		2.0		5.4	11.3		2.5	3.9
RSD (%)		7.0		11.1	14.4		5.4	6.6
	50	51.3	50	74.6	64	50	69.7	58
	50	55.9	50	62.9	74	50	72.2	54
	50	55.5	50	71.9	70	50	72.8	56
Mean		54.2		69.8	69.3		71.6	56.0
STD		2.5		6.1	5.0		1.6	2.0
RSD (%)		4.7		8.8	7.3		2.3	3.6

[a] ND = not detected. The value in parentheses is the estimated limit of detection.

Table 12. Spiked Versus Measured Concentrations of 1,2,3,4,7,8-; 1,2,3,6,7,8-; 2,3,4,6,7,8-; and 1,2,3,7,8,9-HxCDF in Homogenized Human Adipose Lipid Matrix

1,2,3,4,7,8-HxCDF spike level (pg/g)	1,2,3,4,7,8-HxCDF concentration (pg/g)	1,2,3,6,7,8-HxCDF spike level (pg/g)	1,2,3,6,7,8-HxCDF concentration (pg/g)	2,3,4,6,7,8-HxCDF spike level (pg/g)	2,3,4,6,7,8-HxCDF concentration (pg/g)	1,2,3,7,8,9-HxCDF spike level (pg/g)	1,2,3,7,8,9-HxCDF concentration (pg/g)	$^{13}C_{12}$-HxCDF absolute recovery (%)
0	22.0	0	12.3	0	4.9	0	ND (0.5)[a]	55
0	22.1	0	12.4	0	4.2	0	ND (0.7)	57
0	22.4	0	12.7	0	4.2	0	ND (0.9)	52
Mean	22.2		12.4		4.4		ND (0.7)	55.7
STD	0.2		0.2		0.4		0.2	2.5
RSD (%)	1.1		1.7		8.9		25.3	4.6
25	46.7	25	36.6	25	32.3	25	34.8	59
25	52.0	25	38.6	25	33.6	25	28.5	57
25	48.8	25	40.8	25	32.0	25	30.9	60
25	49.0	25	39.8	25	32.5	25	29.2	67
Mean	49.1		39.0		32.6		30.9	60.8
STD	2.2		1.8		0.7		2.8	4.3
RSD (%)	4.4		4.7		2.1		9.1	7.2
62.5	90.2	62.5	83.7	62.5	74.7	62.5	82.7	60
62.5	89.1	62.5	80.5	62.5	75.5	62.5	89.4	63
62.5	90.3	62.5	79.6	62.5	74.2	62.5	82.2	63
62.5	90.7	62.5	76.0	62.5	71.1	62.5	76.3	70
Mean	90.1		79.9		73.9		82.7	64.0
STD	0.7		3.2		2.0		5.4	4.2
RSD (%)	0.7		3.9		2.6		6.5	6.6
125	157.2	125	151.0	125	141.9	125	143.0	57
125	155.4	125	157.7	125	141.6	125	144.1	54
125	156.4	125	149.1	125	151.0	125	163.4	57
Mean	156.3		152.6		144.9		150.1	56.0
STD	0.9		4.5		5.3		11.4	1.7
RSD (%)	0.6		2.9		3.7		7.6	3.1

[a] ND = not detected. The value in parentheses reflects the estimated limit of detection.

Table 13. Spiked Versus Measured Concentration of 1,2,3,4,7,8-; 1,2,3,6,7,8-; and 1,2,3,7,8,9-HxCDD in Homogenized Human Adipose Lipid Samples

	1,2,3,4,7,8-HxCDD spike level (pg/g)	1,2,3,4,7,8-HxCDD concentration (pg/g)	1,2,3,6,7,8-HxCDD spike level (pg/g)	1,2,3,6,7,8-HxCDD concentration (pg/g)	1,2,3,7,8,9-HxCDD spike level (pg/g)	1,2,3,7,8,9-HxCDD concentration (pg/g)	$^{13}C_{12}$-HxCDD absolute recovery (%)
	0	21.6	0	157.0	0	19.1	58
	0	22.7	0	162.0	0	26.0	60
	0	20.3	0	154.0	0	24.7	58
Mean		21.5		157.7		23.2	58.7
STD		1.2		4.0		3.7	1.2
RSD (%)		5.5		2.6		15.8	2.0
	25	47.8	25	184.0	25	63.5	65
	25	52.0	25	165.0	25	46.1	61
	25	53.7	25	198.0	25	40.8	65
	25	52.7	25	193.0	25	57.3	70
Mean		51.6		185.0		51.9	65.3
STD		2.6		14.5		10.4	3.7
RSD (%)		5.1		7.9		20.0	5.6
	62.5	82.9	62.5	220.0	62.5	114.0	64
	62.5	96.7	62.5	239.0	62.5	99.9	66
	62.5	90.3	62.5	220.0	62.5	101.2	66
	62.5	81.9	62.5	220.0	62.5	107.4	77
Mean		87.9		224.8		105.6	68.3
STD		7.0		9.5		6.5	5.9
RSD (%)		7.9		4.2		6.1	8.7
	125	141.1	125	288.0	125	141.3	62
	125	150.8	125	299.0	125	152.7	57
	125	146.0	125	266.0	125	189.4	61
Mean		145.9		284.3		161.1	60.0
STD		4.9		16.8		25.2	2.6
RSD (%)		3.3		5.9		15.6	4.4

Table 14. Spiked Versus Measured Concentrations of 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,7,8,9-HpCDF, and 1,2,3,4,6,7,8-HpCDD in Homogenized Human Adipose Lipid Samples

	1,2,3,4,6,7,8-HpCDF spike level (pg/g)	1,2,3,4,6,7,8-HpCDF concentration (pg/g)	1,2,3,4,7,8,9-HpCDF spike level (pg/g)	1,2,3,4,7,8,9-HpCDF concentration (pg/g)	1,2,3,4,6,7,8-HpCDD spike level (pg/g)	1,2,3,4,6,7,8-HpCDD concentration (pg/g)	$^{13}C_{12}$-HpCDD absolute recovery (%)
	0	30.6	0	ND (1.3)[a]	0	214.7	71
	0	27.3	0	ND (1.1)[a]	0	210.8	74
	0	28.6	0	ND (1.0)[a]	0	215.5	69
Mean		28.9		ND (1.1)[a]		213.7	71.3
STD		1.6		0.2		2.5	2.5
RSD (%)		5.7		13		1.2	3.5
	25	48.6	25	26	25	214.7	83
	25	48.6	25	23	25	239.0	75
	25	51.0	25	23	25	243.9	76
	25	56.3	25	25	25	248.5	78
Mean		51.1		24.1		243.3	78.0
STD		3.6		1.4		4.0	3.6
RSD (%)		7.0		5.7		1.7	4.6
	62.5	86.3	62.5	60	62.5	281.5	77
	62.5	85.7	62.5	60	62.5	288.1	84
	62.5	80.9	62.5	59	62.5	269.9	90
	62.5	83.4	62.5	58	62.5	274.9	99
Mean		84.1		59.0		278.6	87.5
STD		2.5		0.8		8.0	9.3
RSD (%)		2.9		1.3		2.9	10.7
	125	154.5	125	119	125	353.2	62
	125	157.3	125	126	125	355.4	61
	125	154.4	125	121	125	343.7	69
		155.4		122.4		350.8	64
		1.6		3.7		6.2	4.4
		1.0		3.0		1.8	6.8

[a] ND = not detected. The value in parentheses is the estimated limit of detection.

Table 15. Spiked Versus Measured Concentrations of OCDF and OCDD in Homogenized Human Adipose Lipid Samples

OCDF spike level (pg/g)	OCDF concentration (pg/g)	OCDD spike level (pg/g)	OCDD concentration (pg/g)	$^{13}C_{12}$-OCDD abolute recovery (%)
0	4.9	0	804	91
0	2.3	0	833	88
0	2.6	0	781	94
Mean	3.2		806.1	91.0
STD	1.4		26.1	3.0
RSD (%)	43.1		3.2	3.3
50	44.2	50	849	100
50	45.0	50	856	87
50	45.9	50	876	91
50	49.6	50	860	91
Mean	46.2		860.4	92.3
STD	2.4		11.4	5.5
RSD (%)	5.2		1.3	6.0
125	111.1	125	932	90
125	107.8	125	934	96
125	110.7	125	944	102
125	113.1	125	907	104
Mean	110.7		929.1	98.0
STD	2.2		15.7	6.3
RSD (%)	2.0		1.7	6.5
250	227.8	250	1,080	69
250	231.0	250	1,140	67
250	228.6	250	1,070	74
Mean	229.1		1,096	70.0
STD	1.7		34.7	3.6
RSD (%)	0.7		3.2	5.2

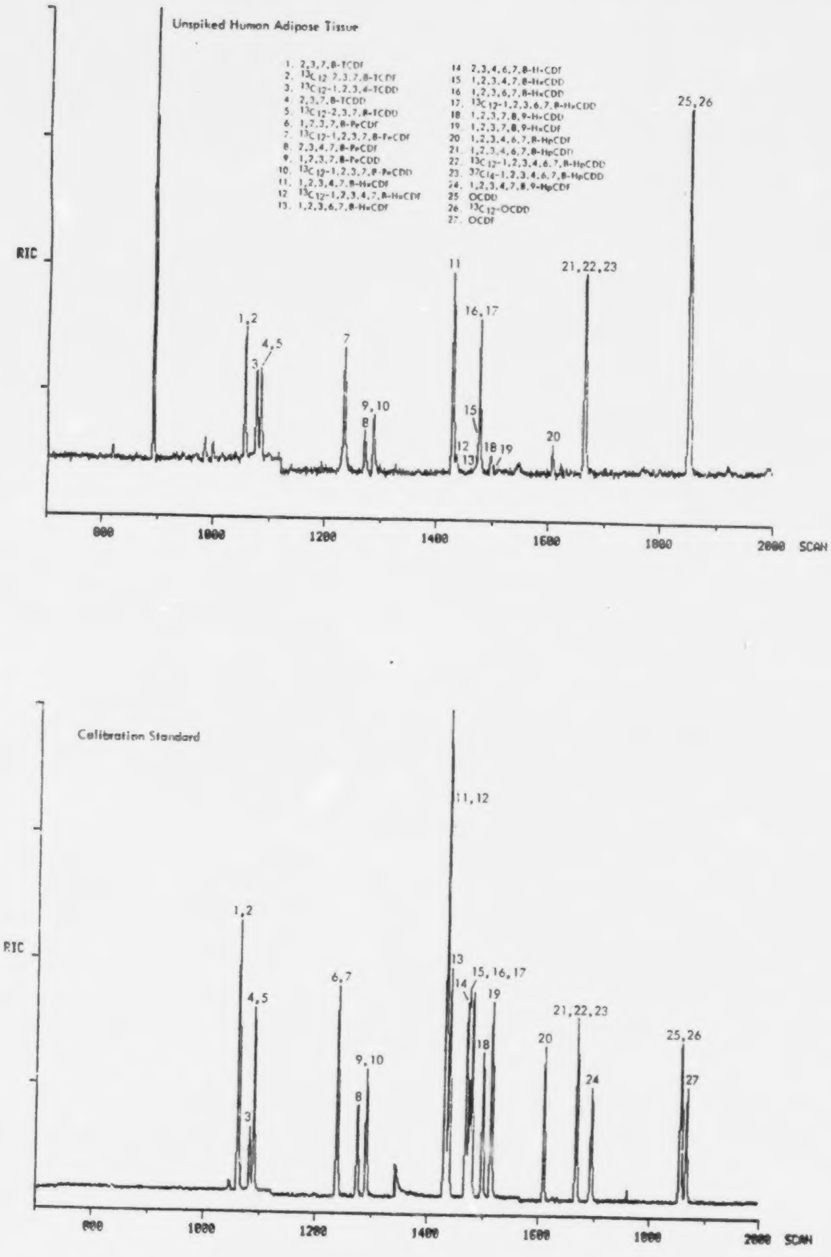

Figure 1. Comparison of the HRGC/MS-SIM reconstructed ion chromatogram (RIC) from the analysis of unspiked homogenized human adipose tissue matrix and a calibration standard for PCDDs and PCDFs.

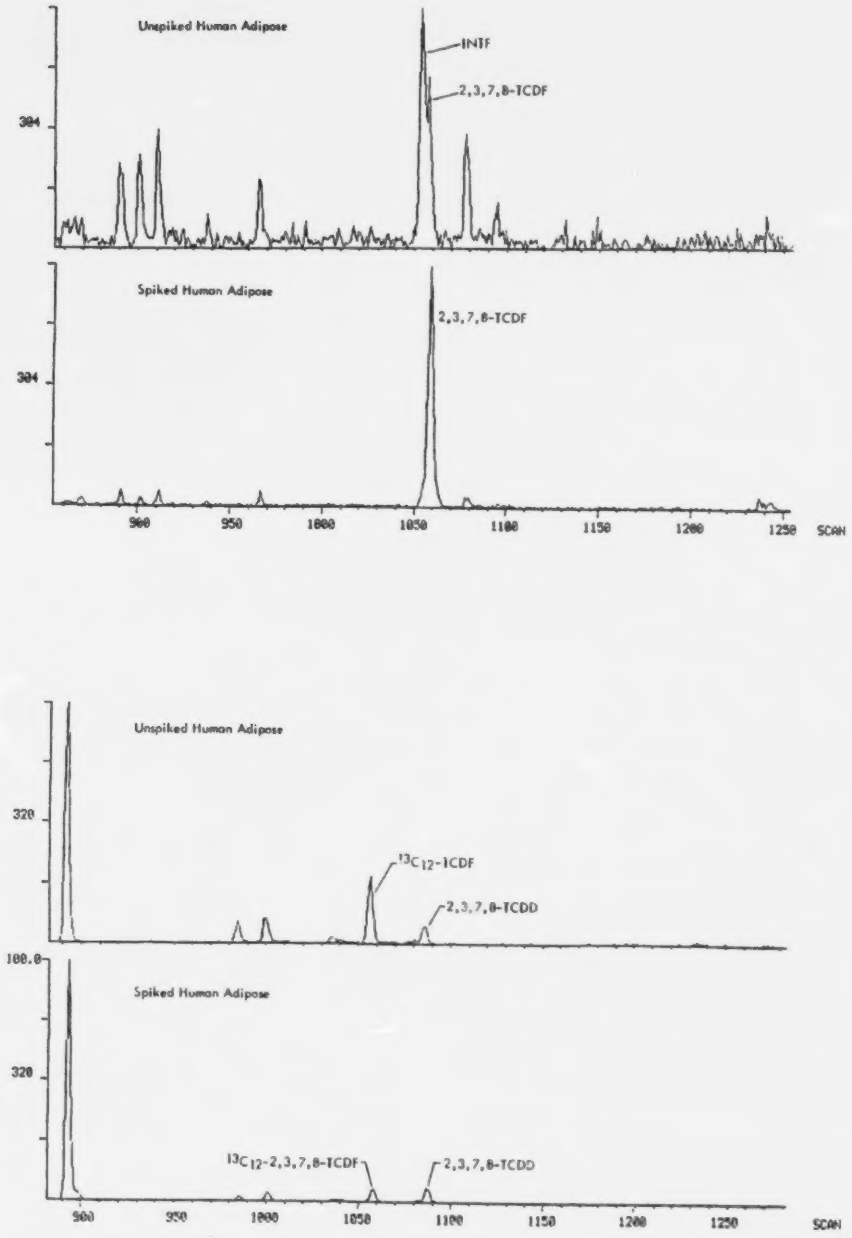

Figure 2. Example of the TCDF (m/z 304) and TCDD (m/z 320) HRGC/MS-SIM elution profiles in unspiked and spiked human adipose. The spiked concentrations for TCDF and TCDD in these chromatograms were 25 pg/g each.

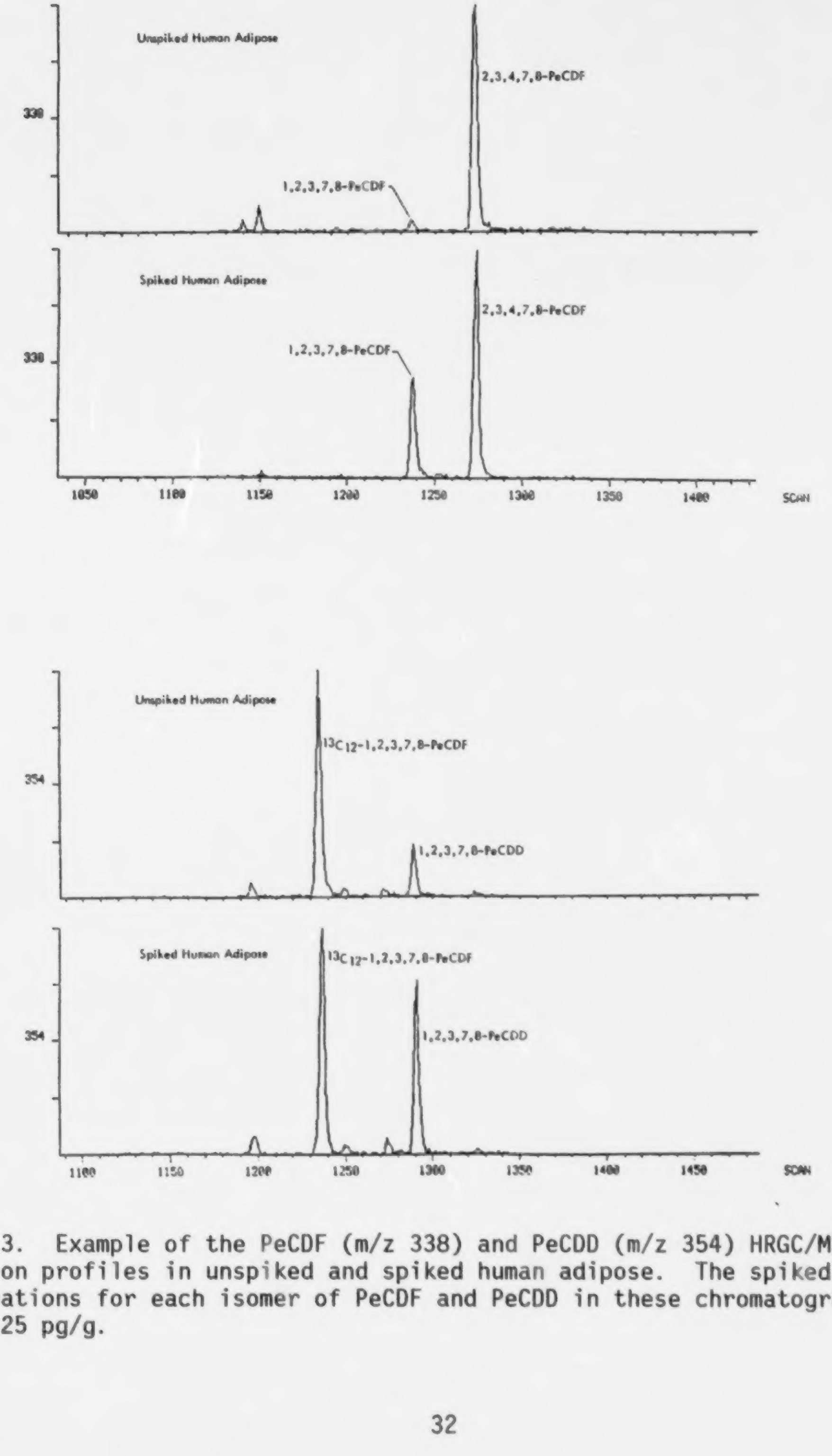

Figure 3. Example of the PeCDF (m/z 338) and PeCDD (m/z 354) HRGC/MS-SIM elution profiles in unspiked and spiked human adipose. The spiked concentrations for each isomer of PeCDF and PeCDD in these chromatograms were 25 pg/g.

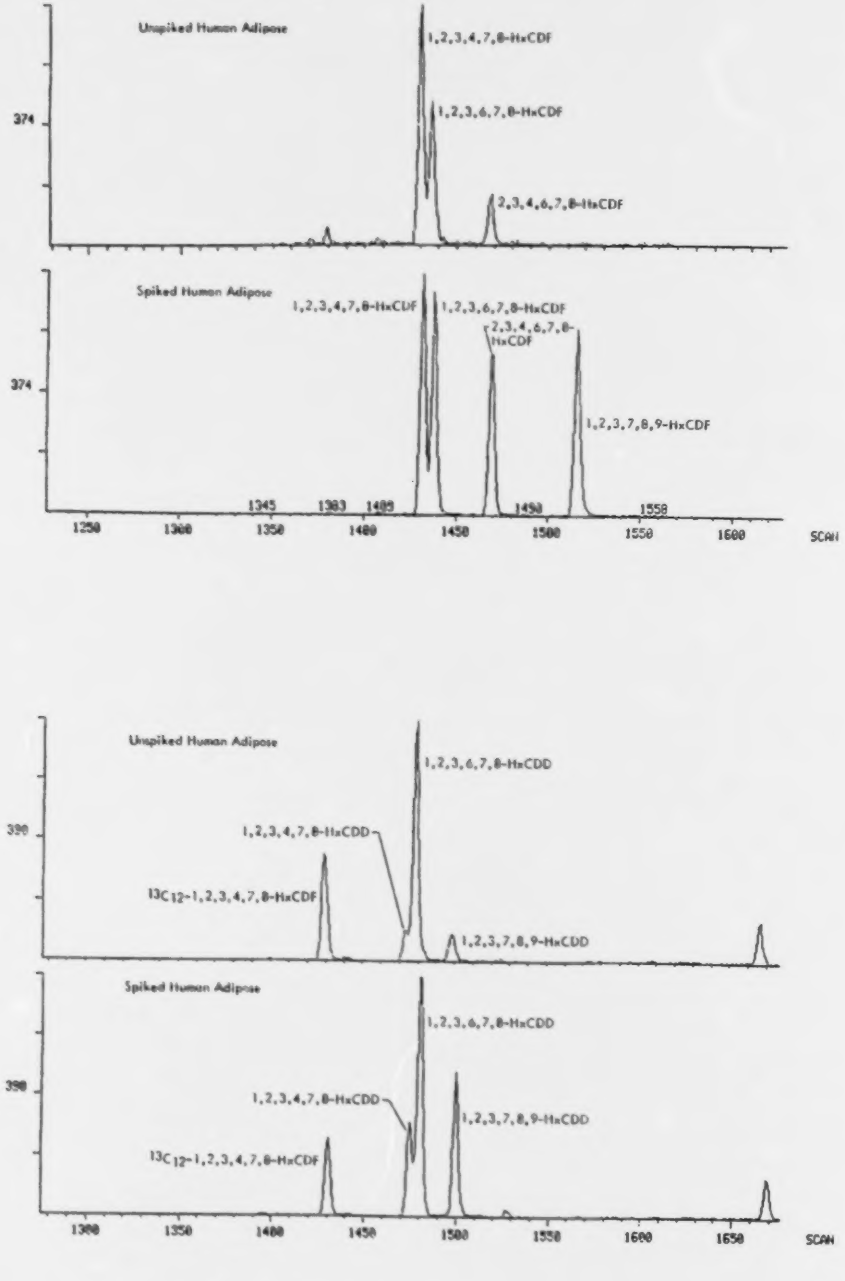

Figure 4. Example of the HxCDF (m/z 374) and HxCDD (m/z 390) HRGC/MS-SIM elution profiles in unspiked and spiked human adipose. The spiked concentrations for each isomer of HxCDF and HxCDD were 62.5 pg/g.

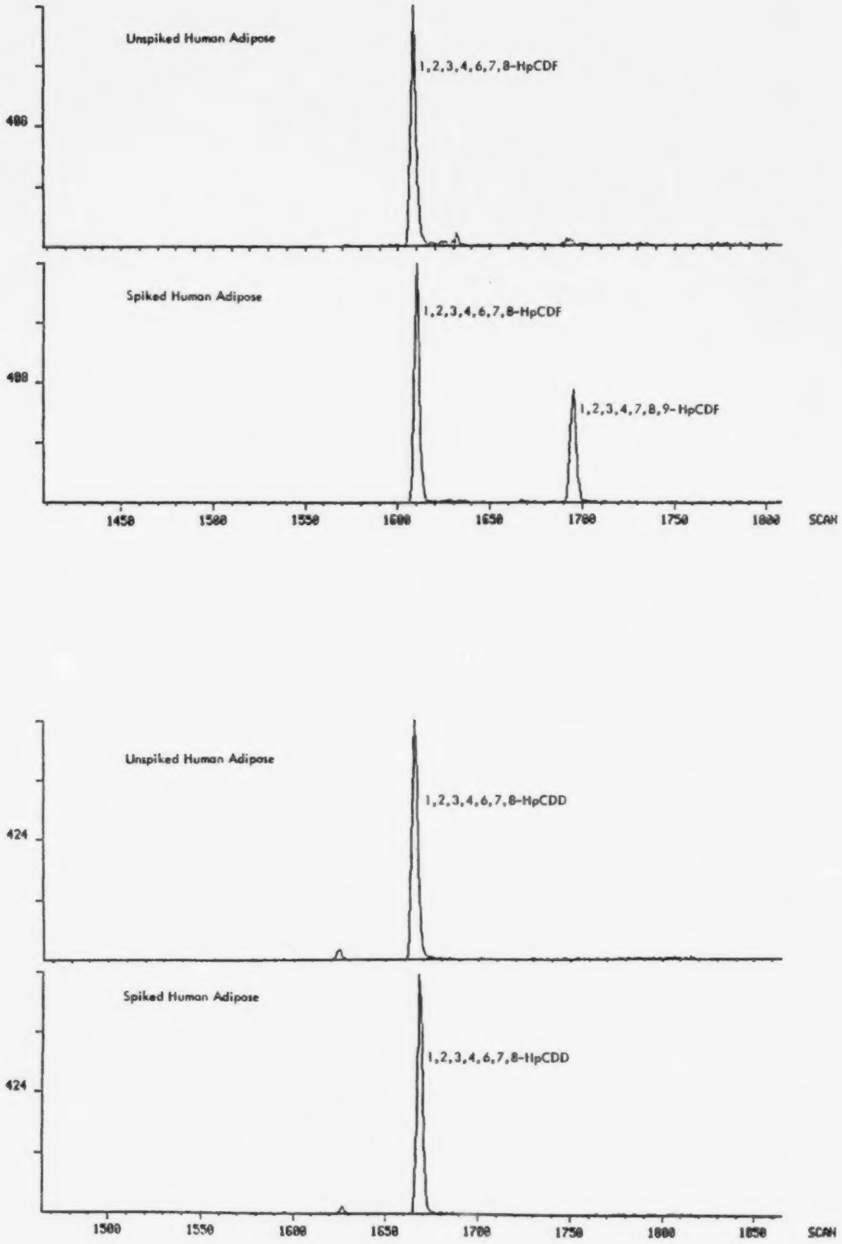

Figure 5. Example of the HpCDF (m/z 408) and HpCDD (m/z 424) HRGC/MS-SIM elution profiles in unspiked and spiked human adipose. The spiked concentrations for each isomer of HpCDF and HpCDD were 62.5 pg/g.

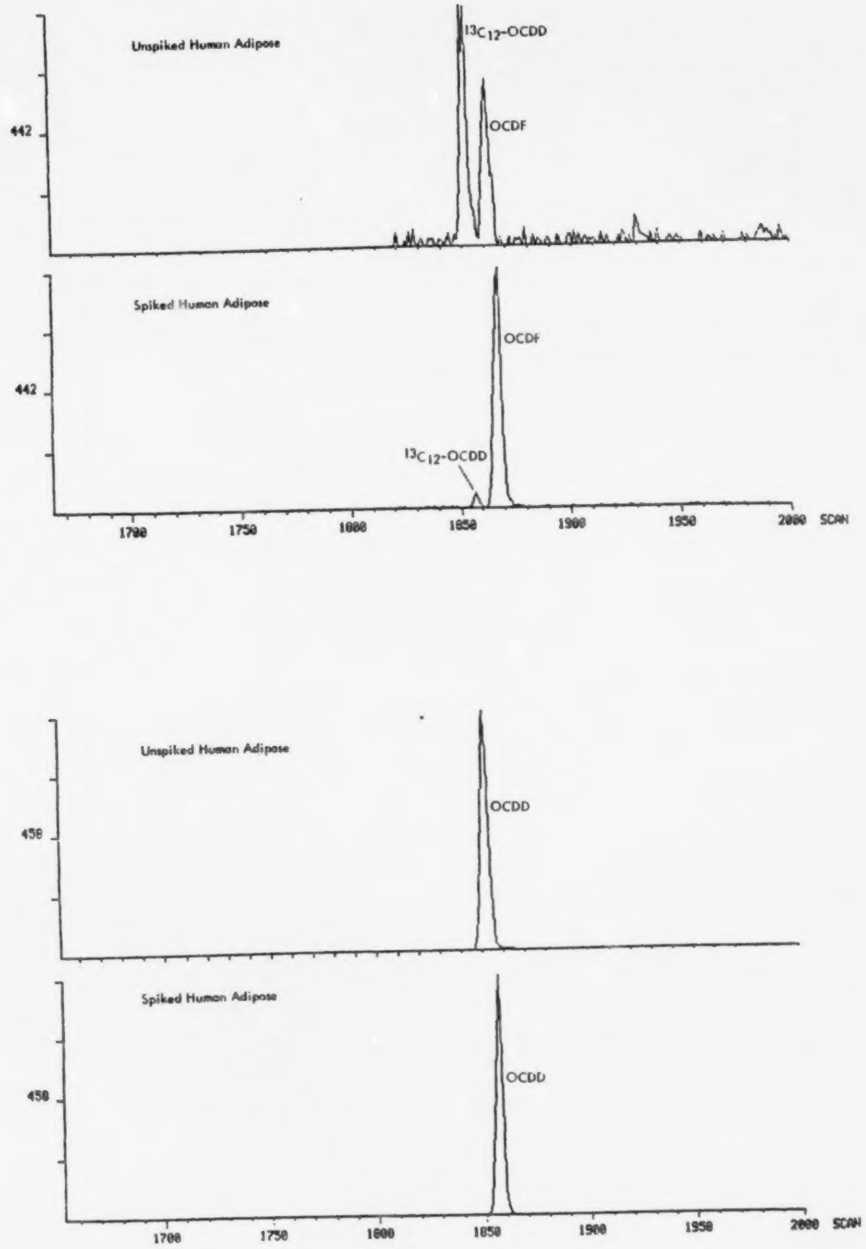

Figure 6. Examples of the OCDF (m/z 442) and OCDD (m/z 458) HRGC/MS-SIM elution profiles in unspiked and spiked human adipose. The spiked concentrations for OCDD and OCDF were 125 pg/g each.

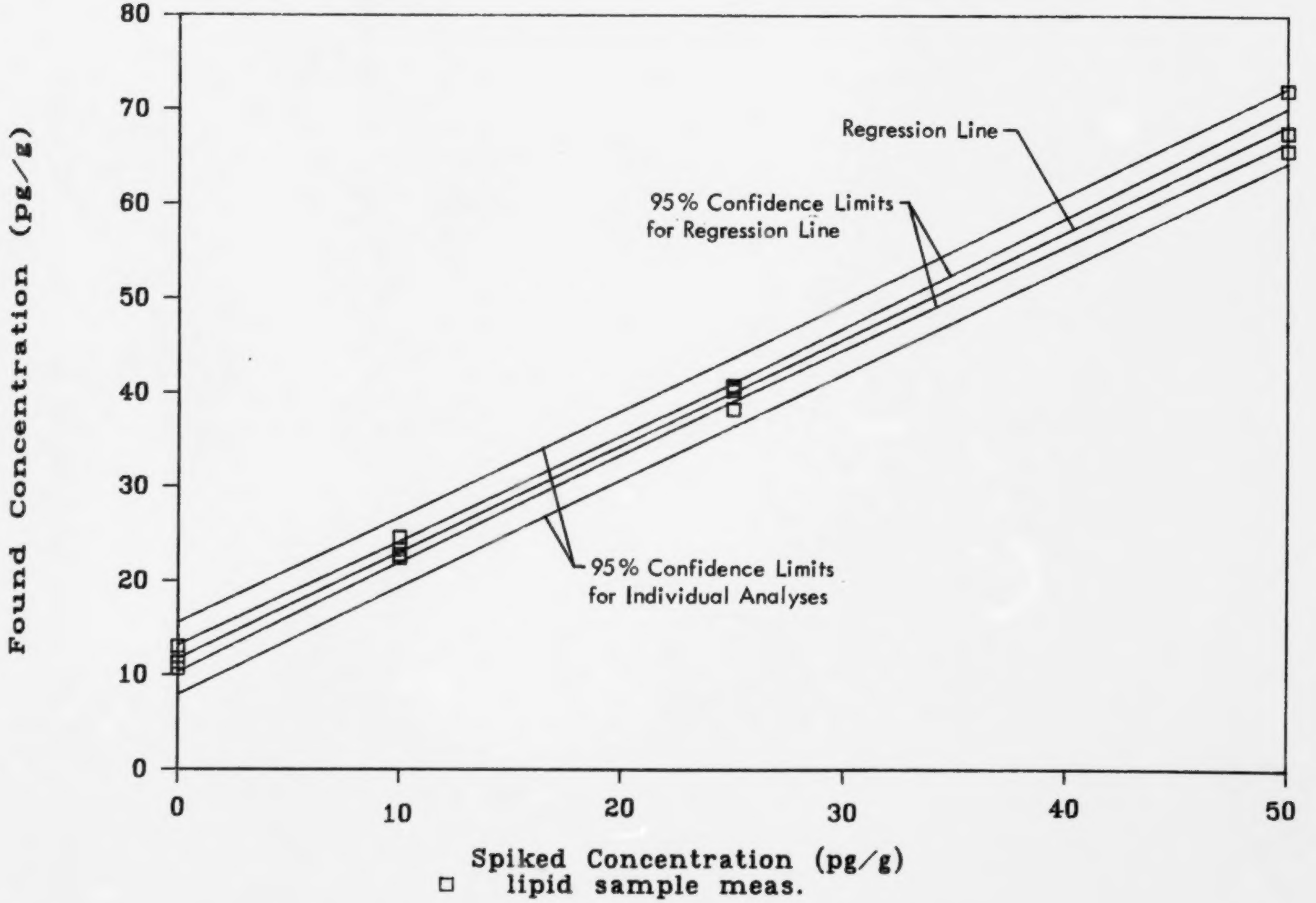

Figure 7. Measured concentrations versus concentrations of 2,3,7,8-TCDD spiked into the homogenized human adipose lipid matrix.

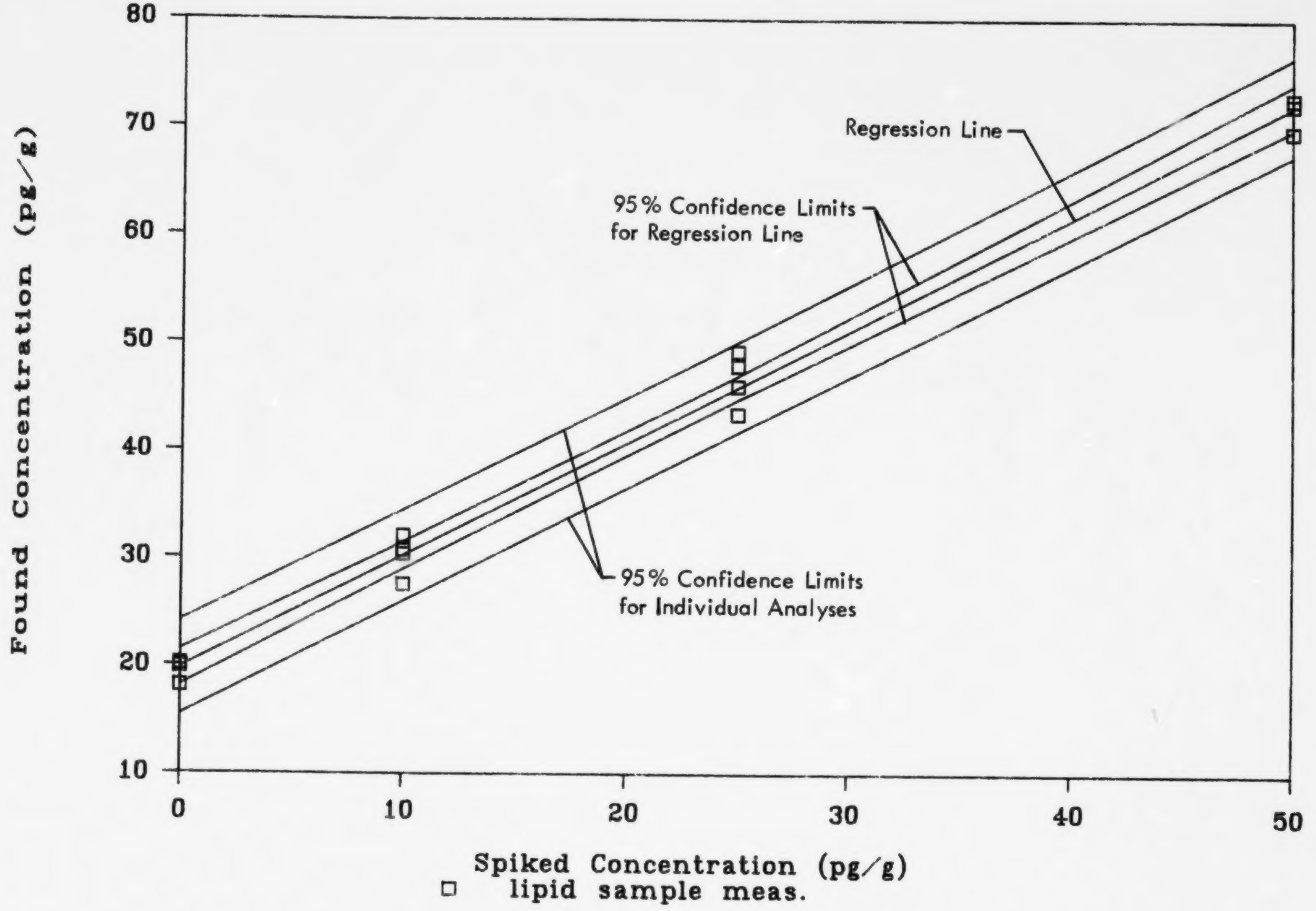

Figure 8. Measured concentrations versus concentrations of 1,2,3,7,8-PeCDD spiked into the homogenized human adipose lipid matrix.

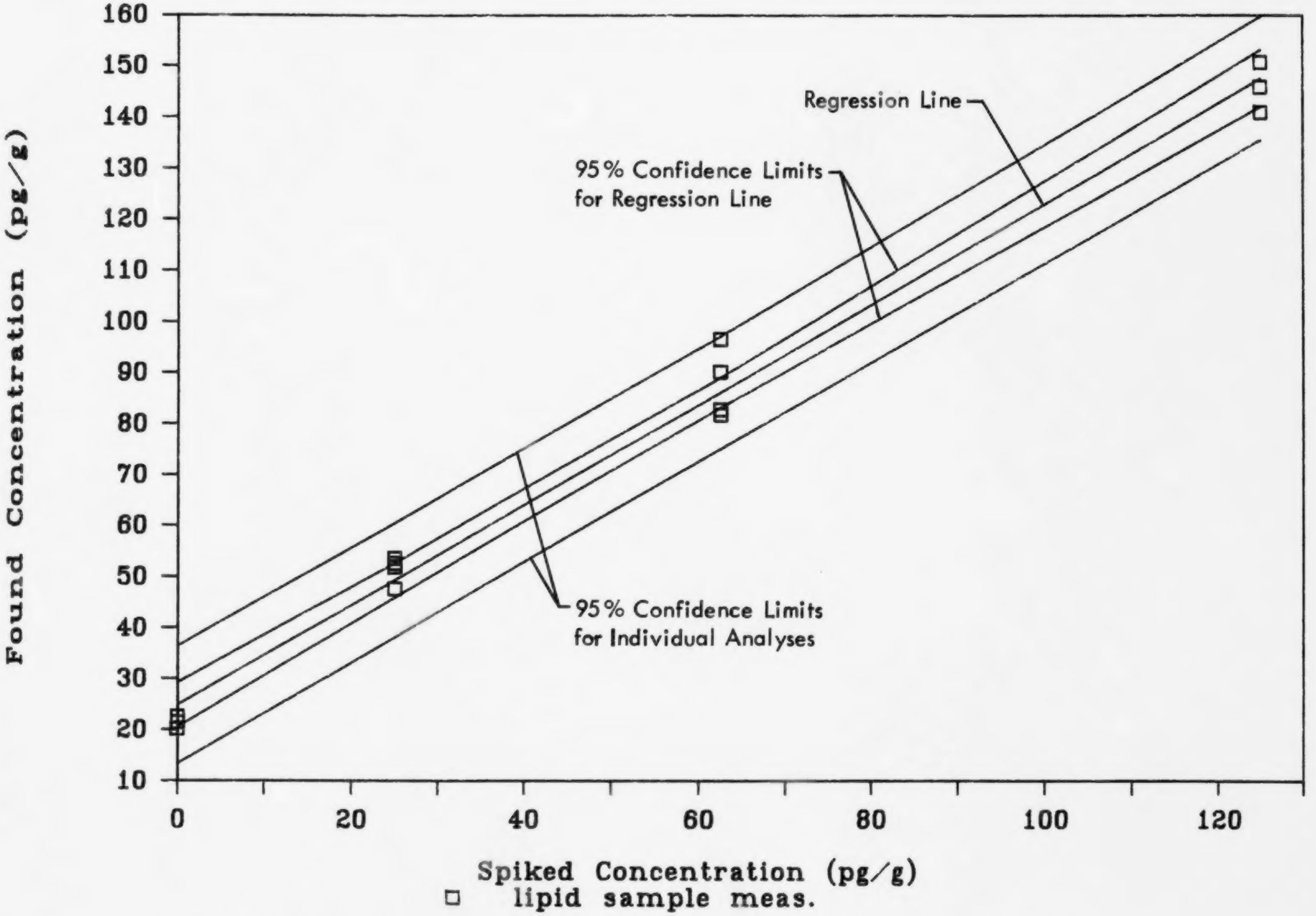

Figure 9. Measured concentrations versus concentrations of 1,2,3,4,7,8-HxCDD spiked into the homogenized human adipose lipid matrix.

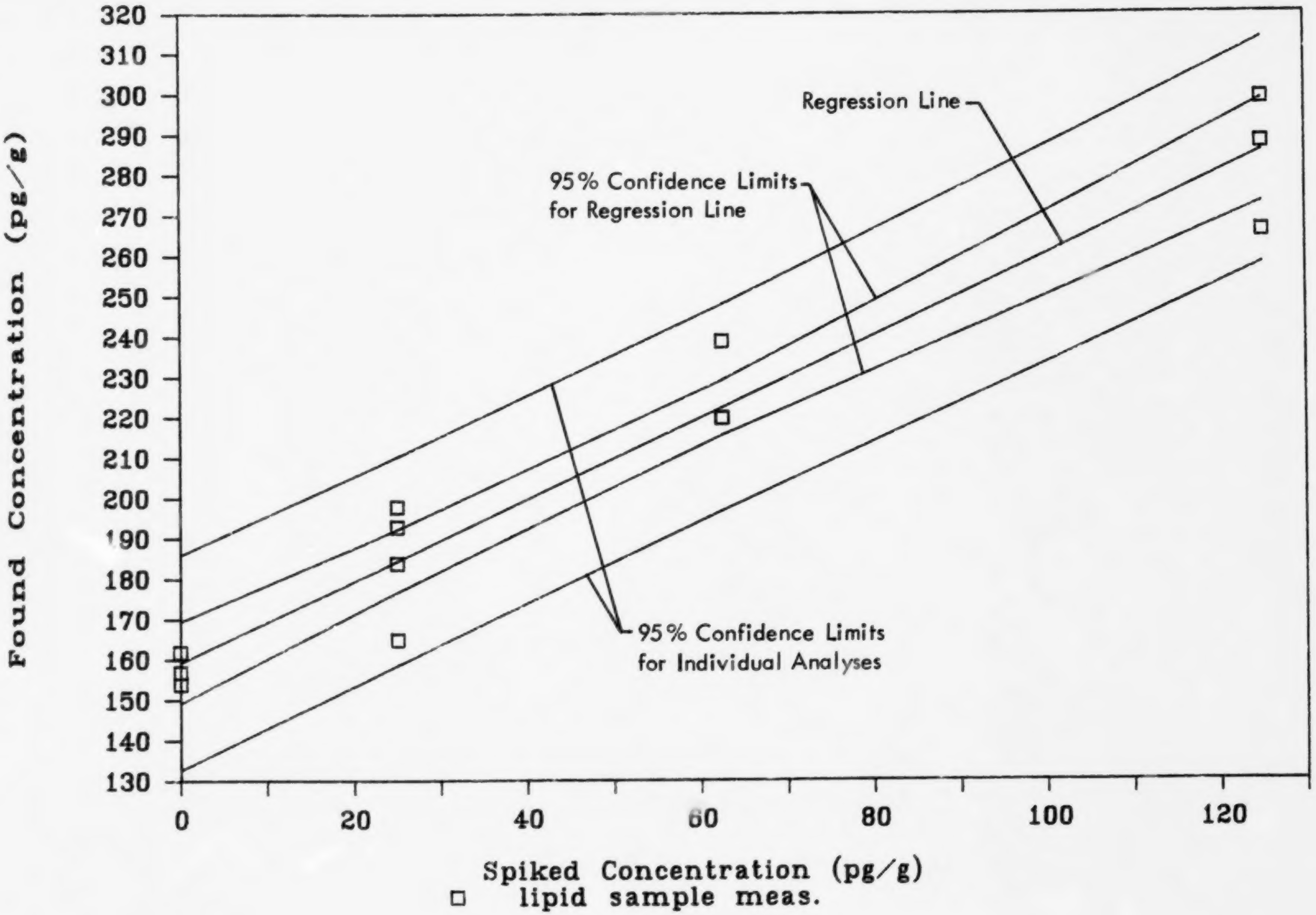

Figure 10. Measured concentrations versus concentrations of 1,2,3,6,7,8-HxCDD spiked into the homogenized human adipose lipid matrix.

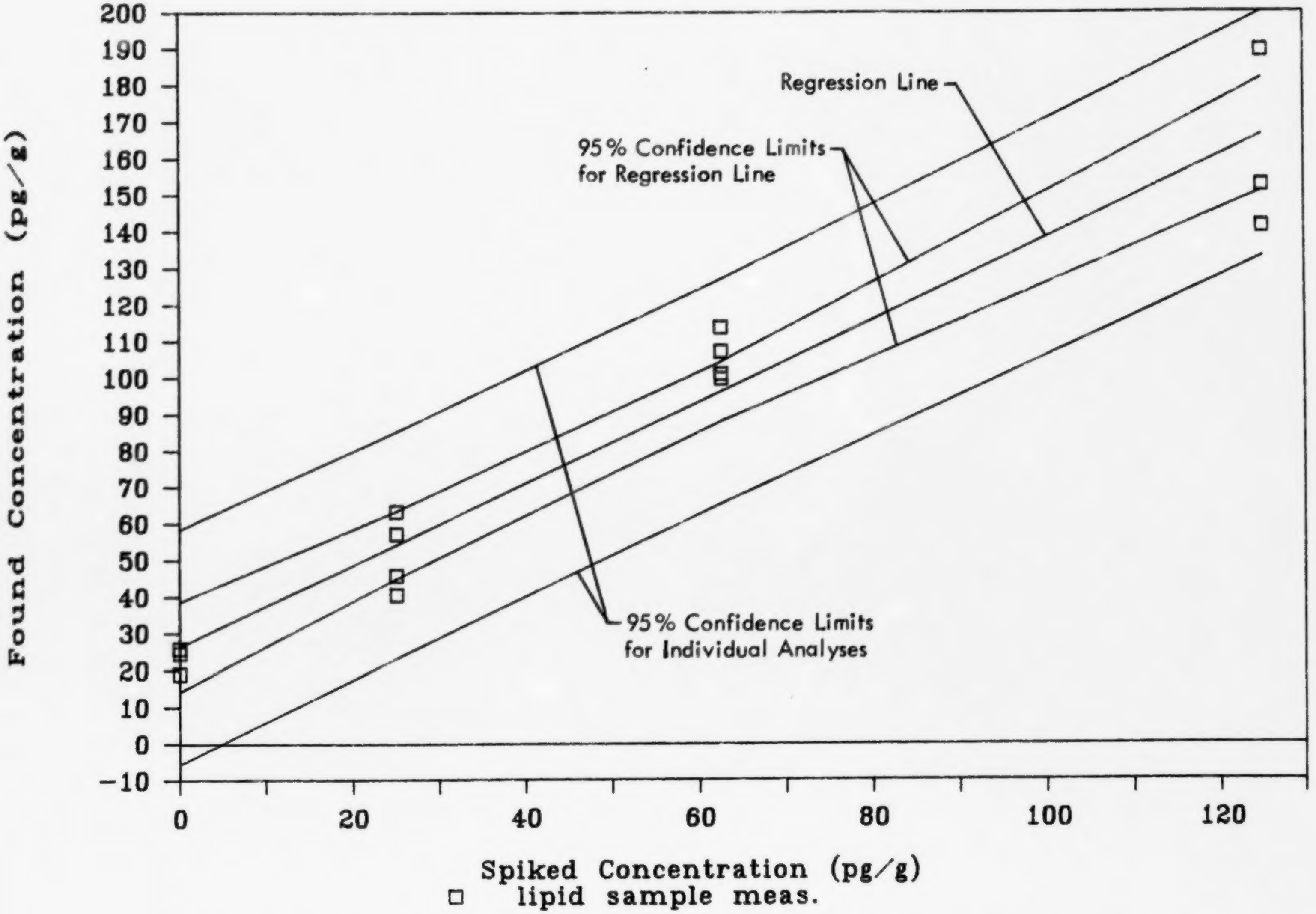

Figure 11. Measured concentrations versus concentrations of 1,2,3,7,8,9-HxCDD spiked into the homogenized human adipose lipid matrix.

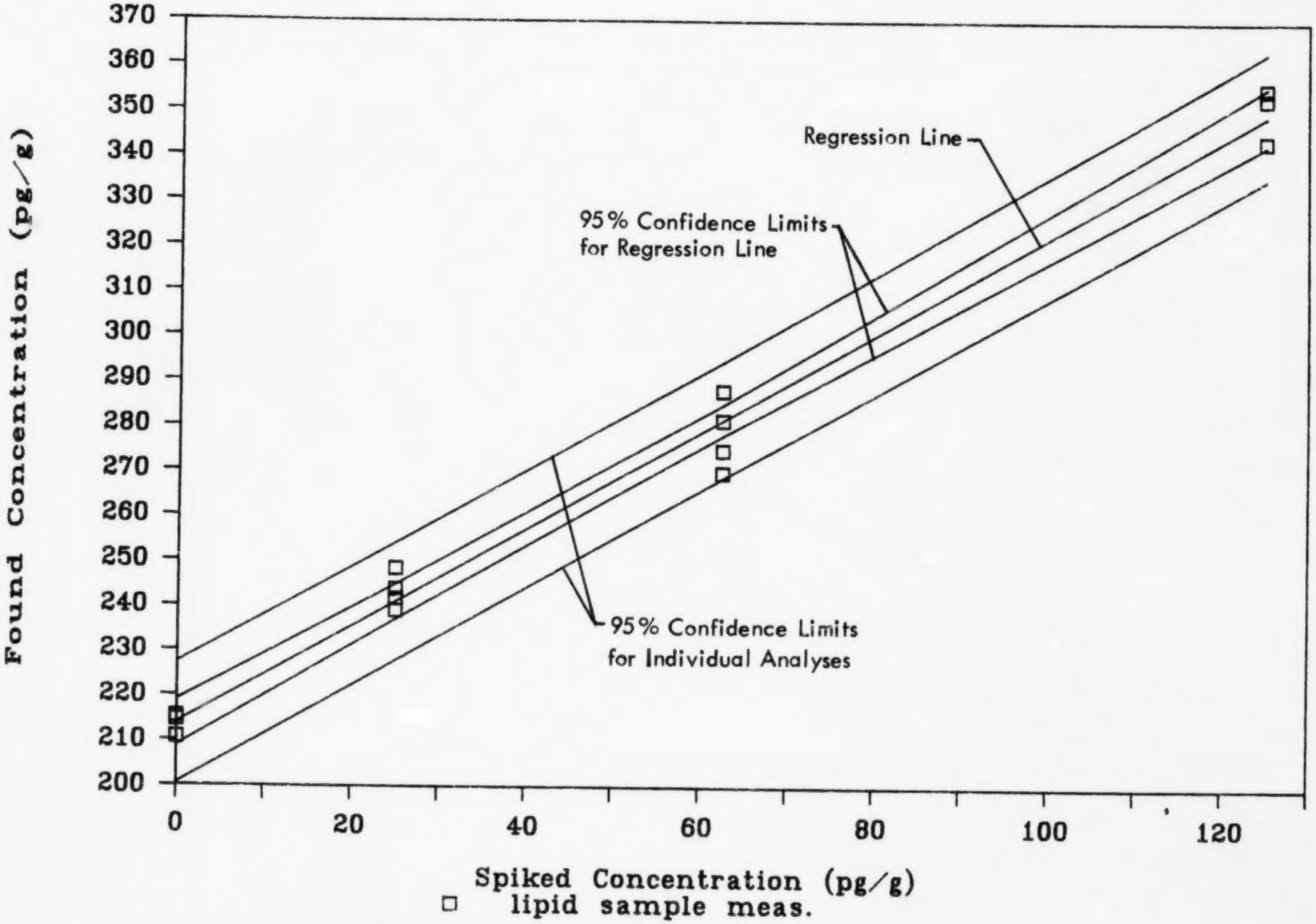

Figure 12. Measured concentrations versus concentrations of 1,2,3,4,6,7,8-HpCDD spiked into the homogenized human adipose lipid matrix.

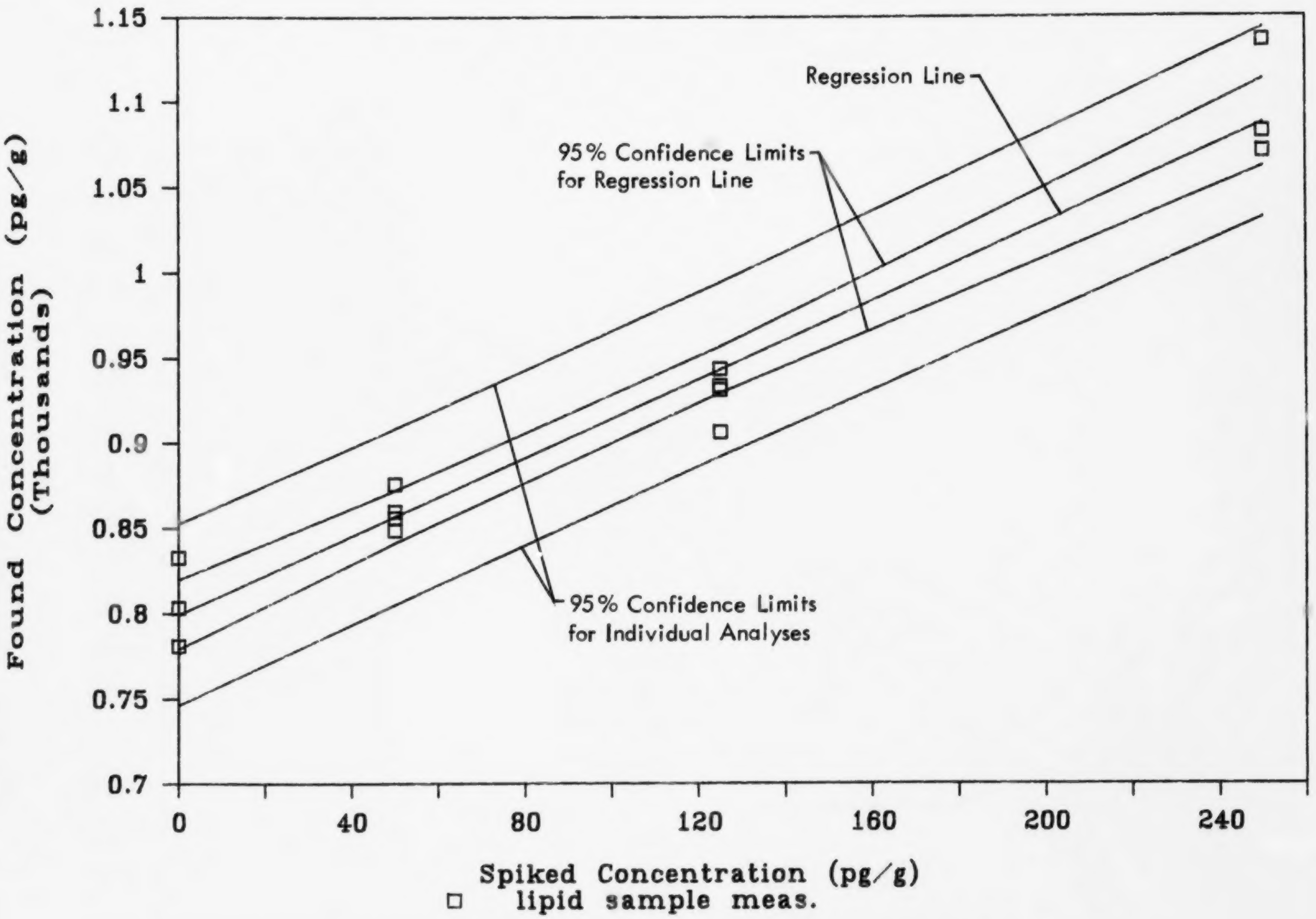

Figure 13. Measured concentrations versus concentrations of OCDD spiked into the homogenized human adipose lipid matrix.

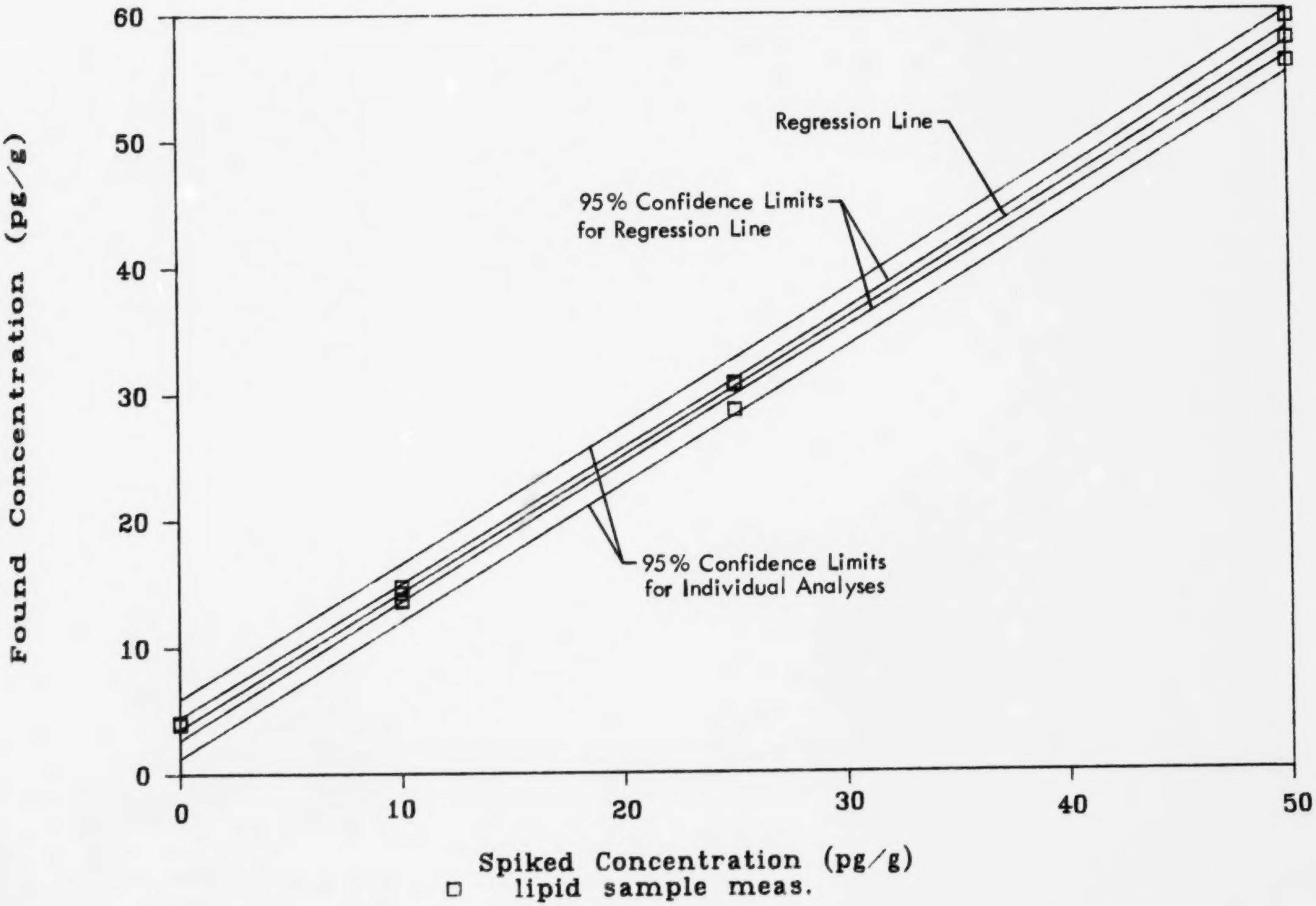

Figure 14. Measured concentrations versus concentrations of 2,3,7,8-TCDF spiked into the homogenized human adipose lipid matrix.

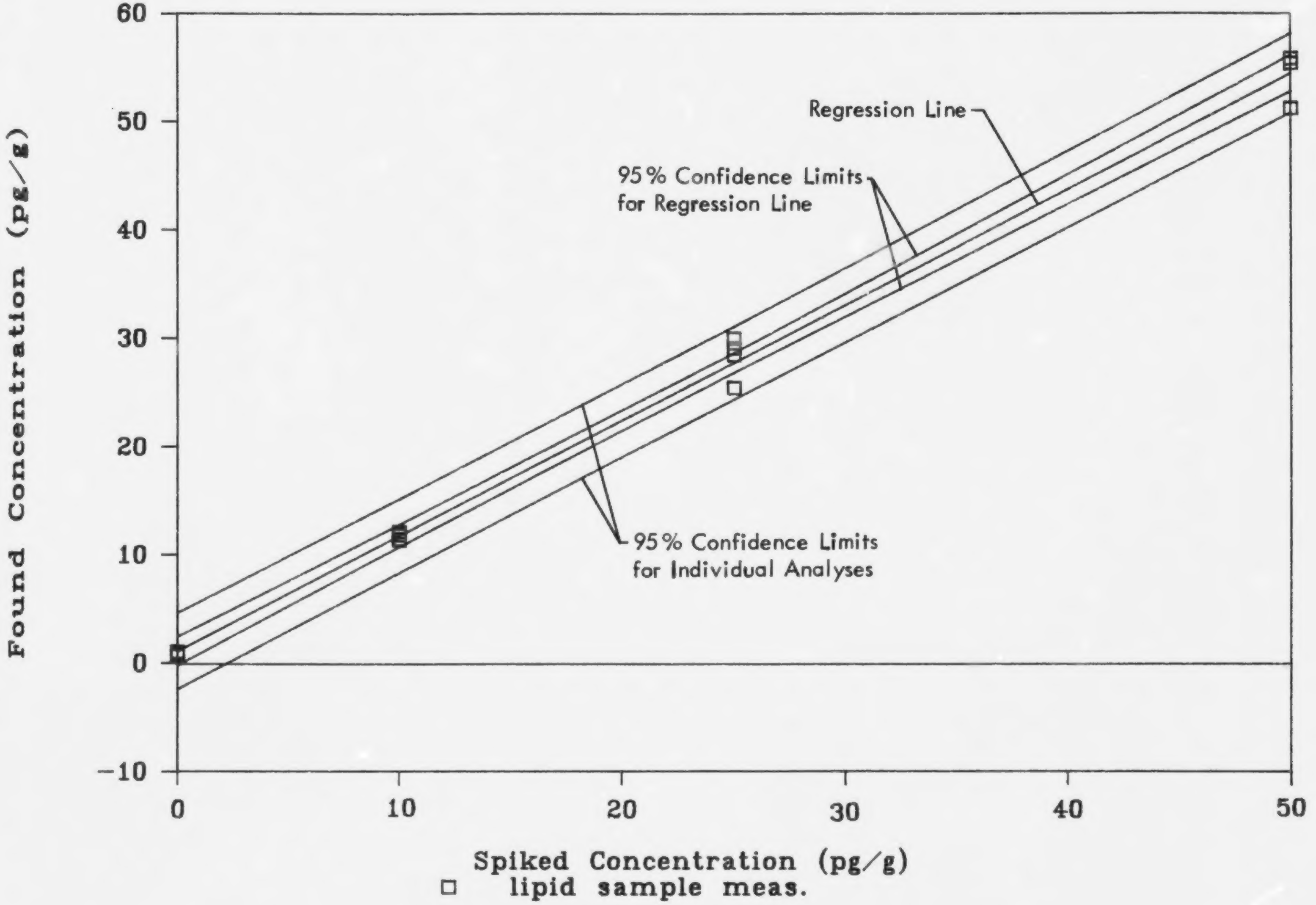

Figure 15. Measured concentrations versus concentrations of 1,2,3,7,8-PeCDF spiked into the homogenized human adipose lipid matrix.

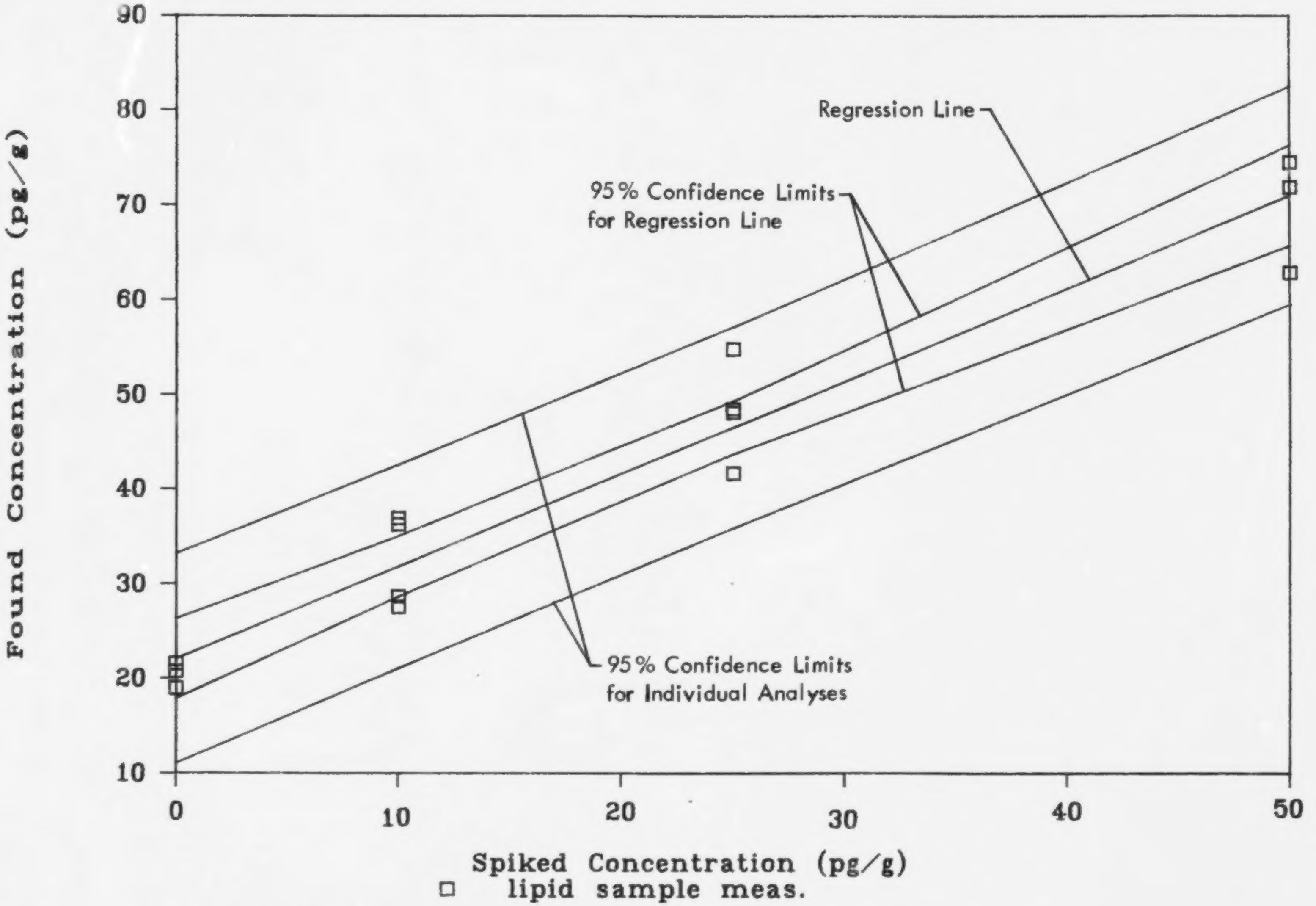

Figure 16. Measured concentrations versus concentrations of 2,3,4,7,8-PeCDF spiked into the homogenized human adipose lipid matrix.

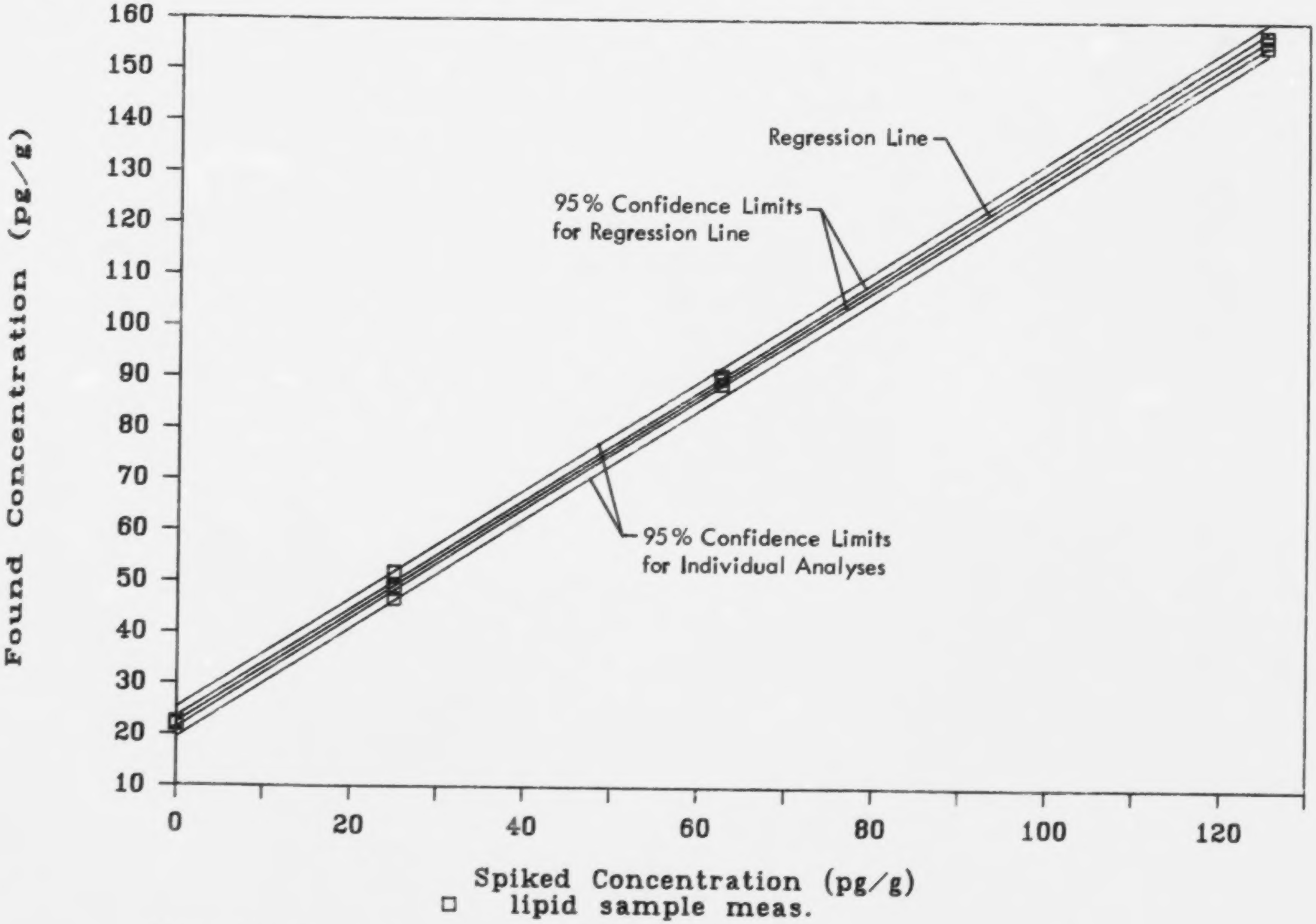

Figure 17. Measured concentrations versus concentrations of 1,2,3,4,7,8-HxCDF spiked into the homogenized human adipose lipid matrix.

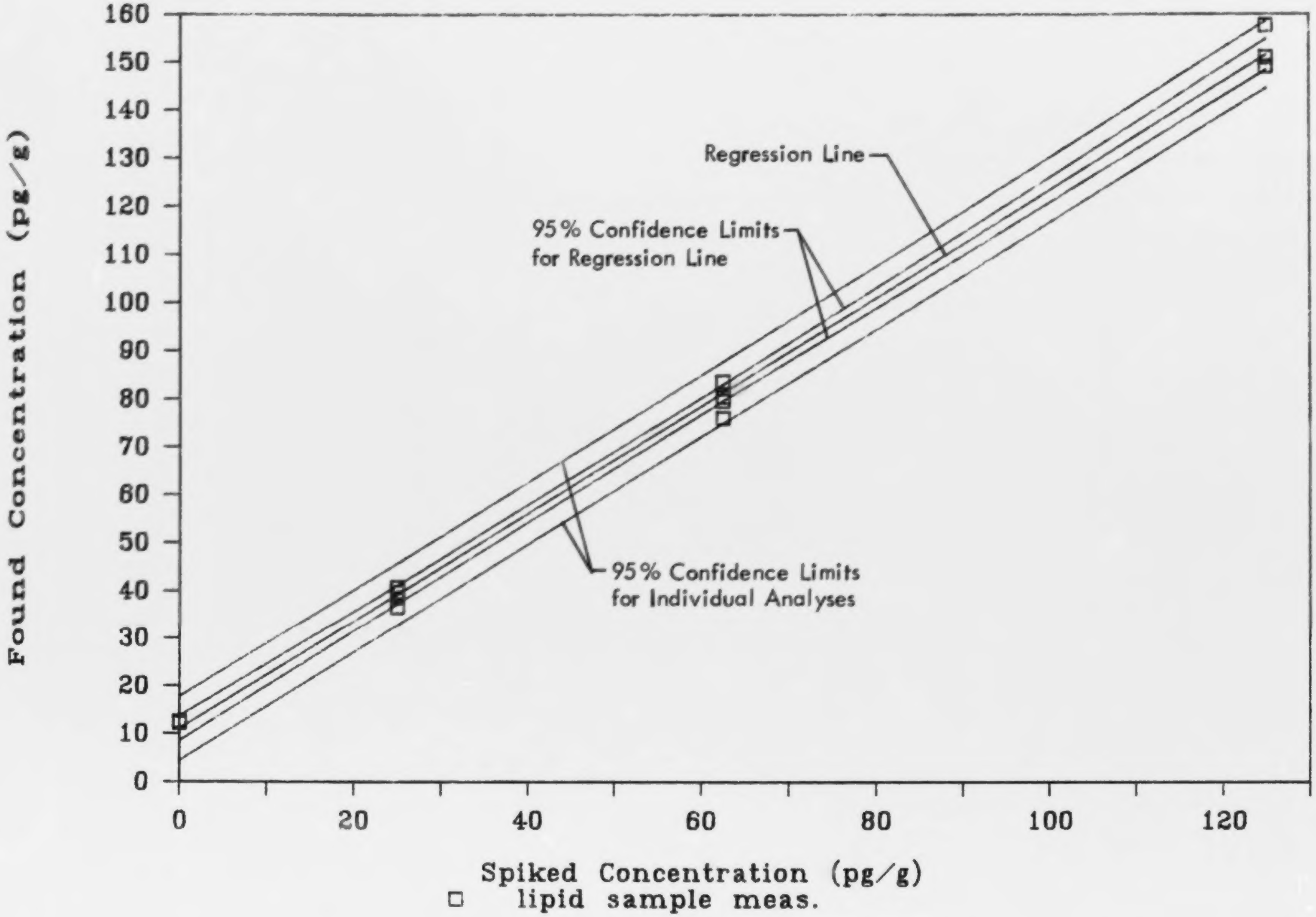

Figure 18. Measured concentrations versus concentrations of 1,2,3,6,7,8-HxCDF spiked into the homogenized human adipose lipid matrix.

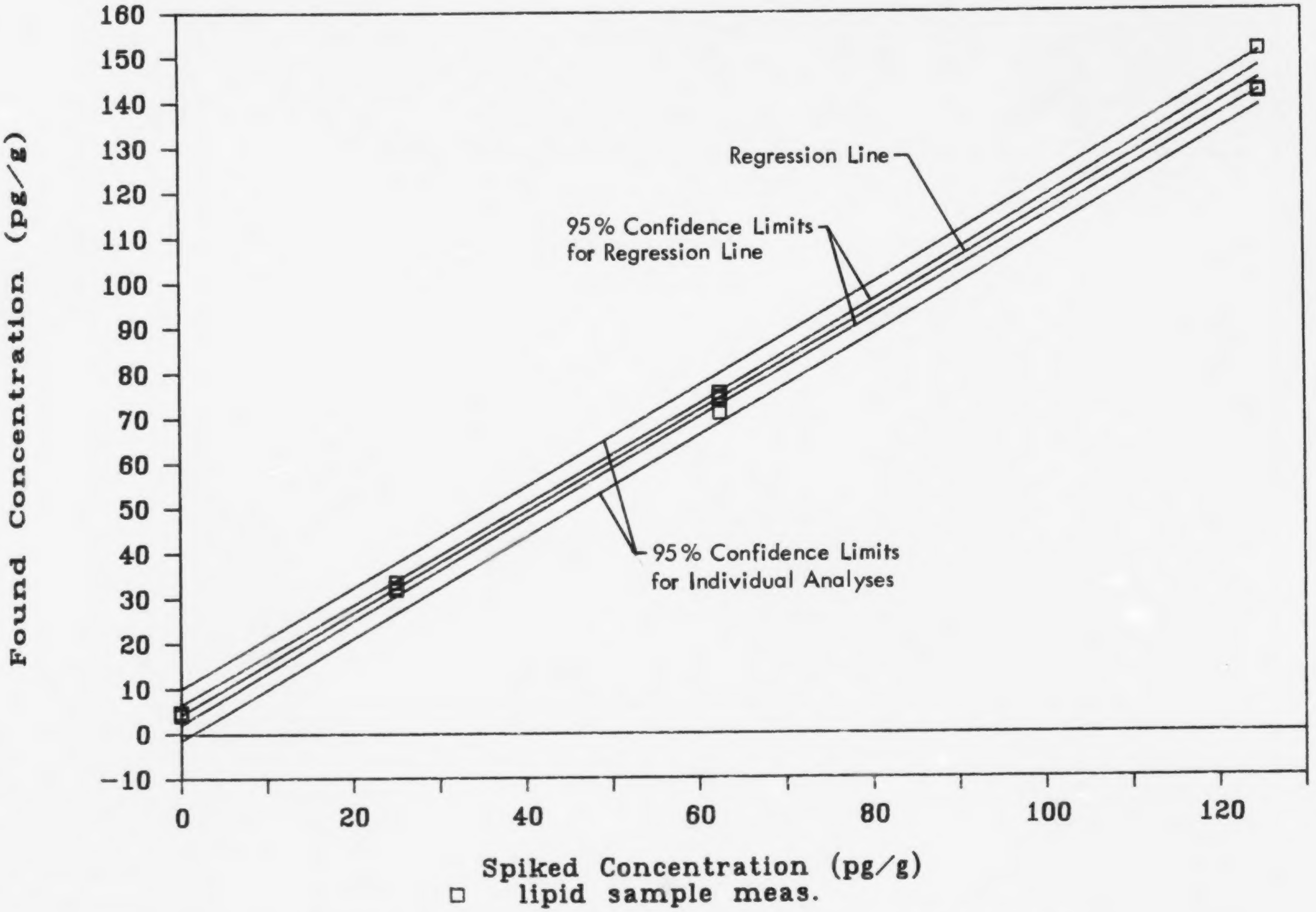

Figure 19. Measured concentrations versus concentrations of 2,3,4,6,7,8-HxCDF spiked into the homogenized human adipose lipid matrix.

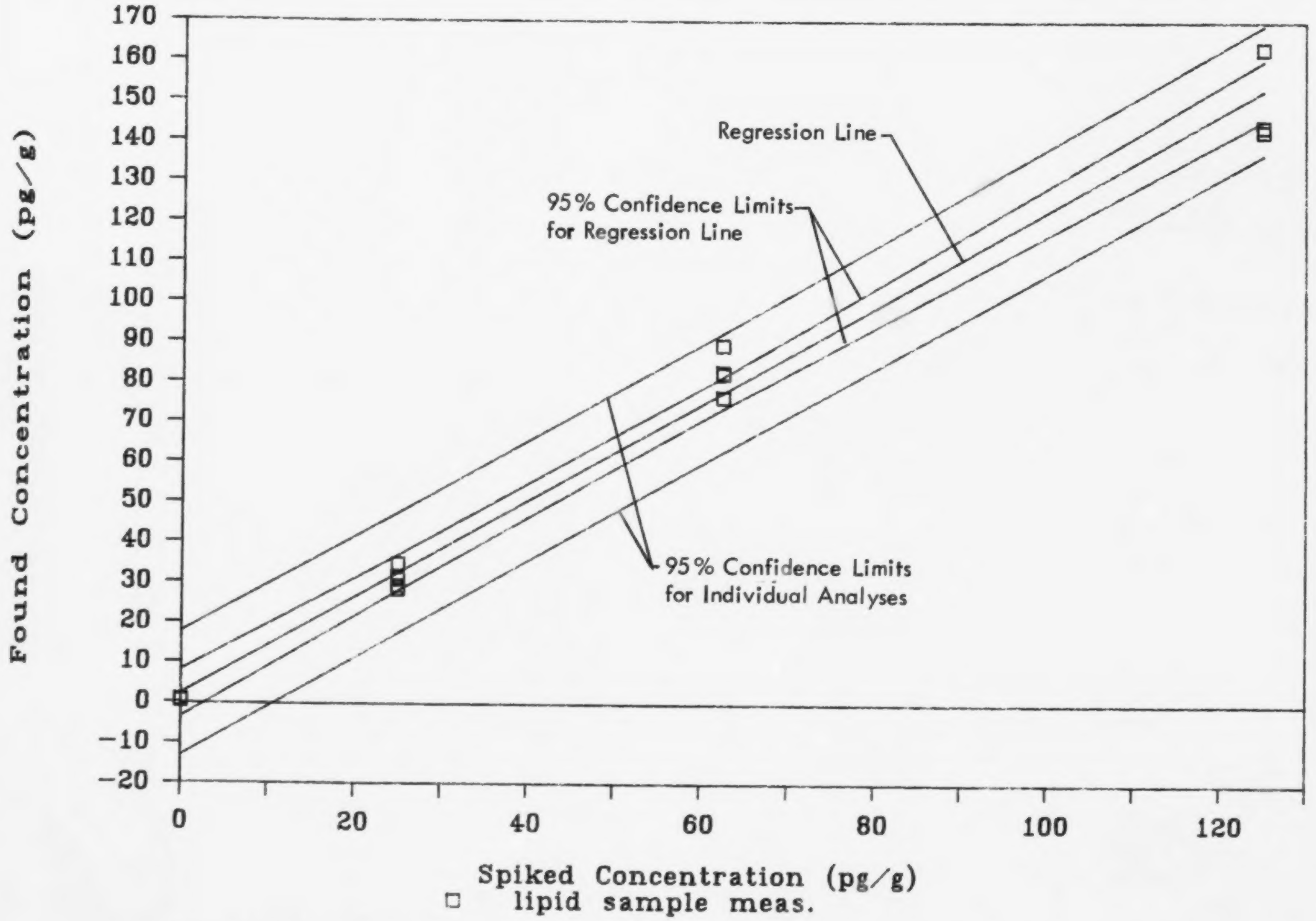

Figure 20. Measured concentrations versus concentrations of 1,2,3,7,8,9-HxCDF spiked into the homogenized human adipose lipid matrix.

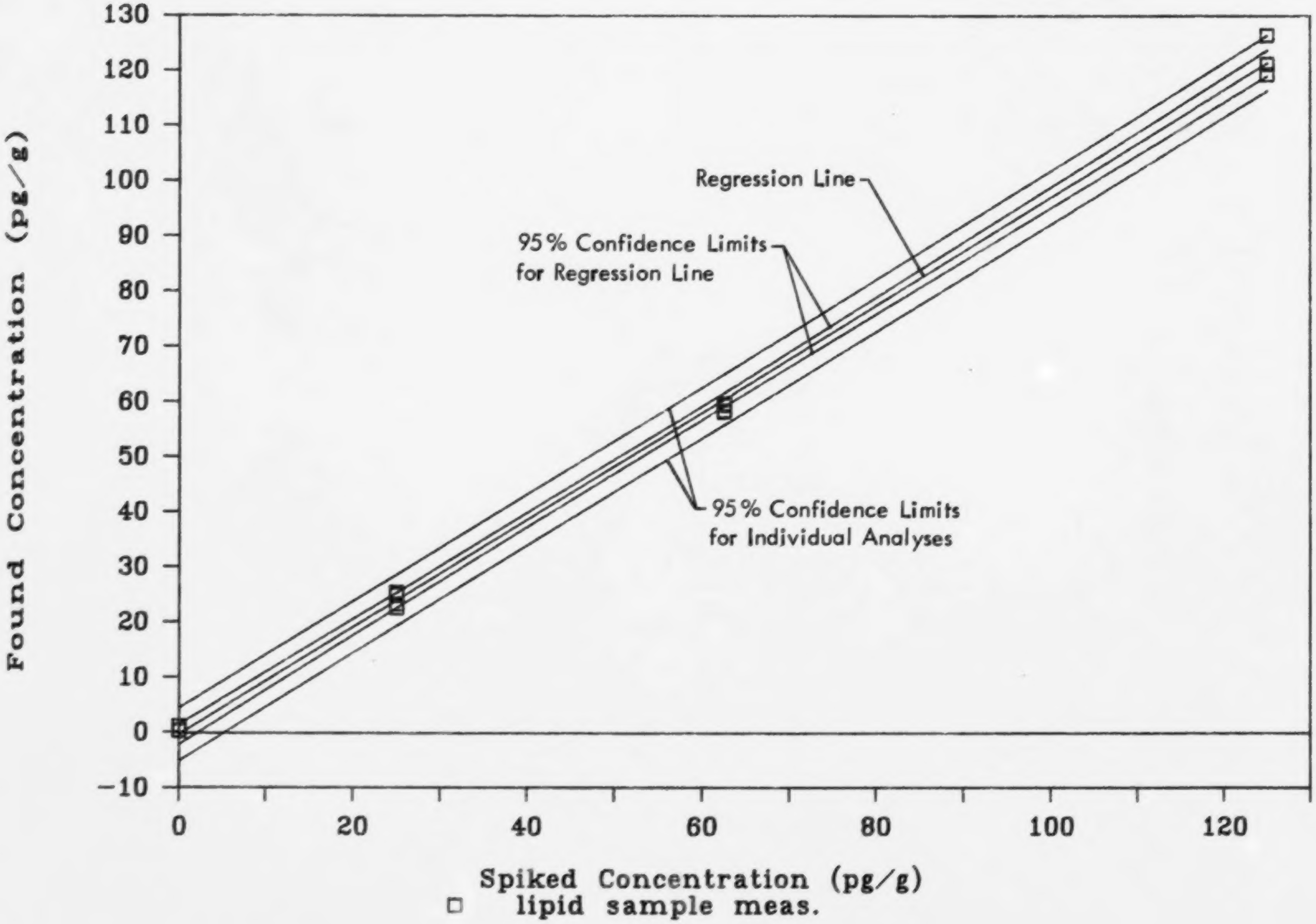

Figure 21. Measured concentrations versus concentrations of 1,2,3,4,7,8,9-HpCDF spiked into the homogenized human adipose lipid matrix.

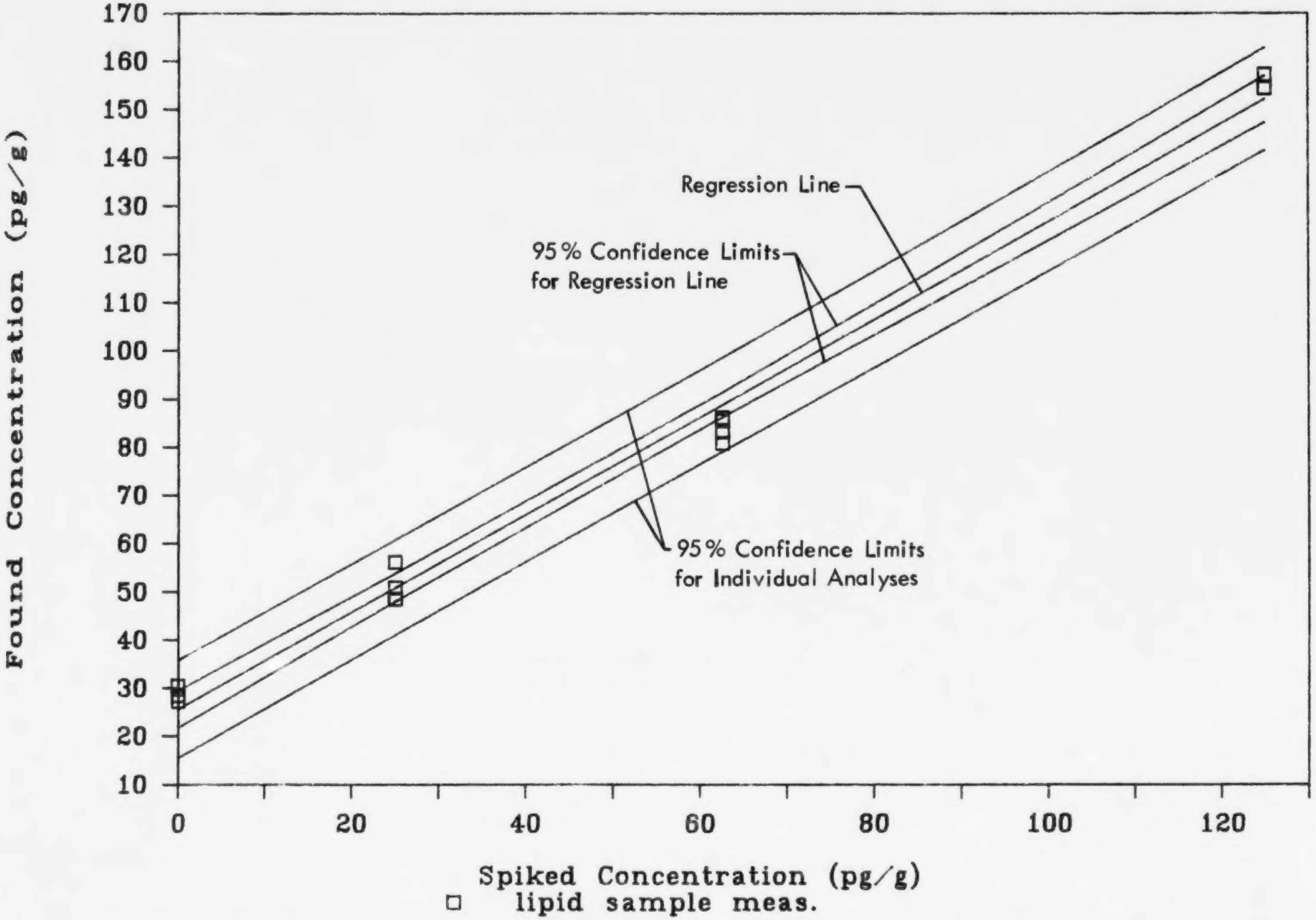

Figure 22. Measured concentrations versus concentrations of 1,2,3,4,6,7,8-HpCDF spiked into the homogenized human adipose lipid matrix.

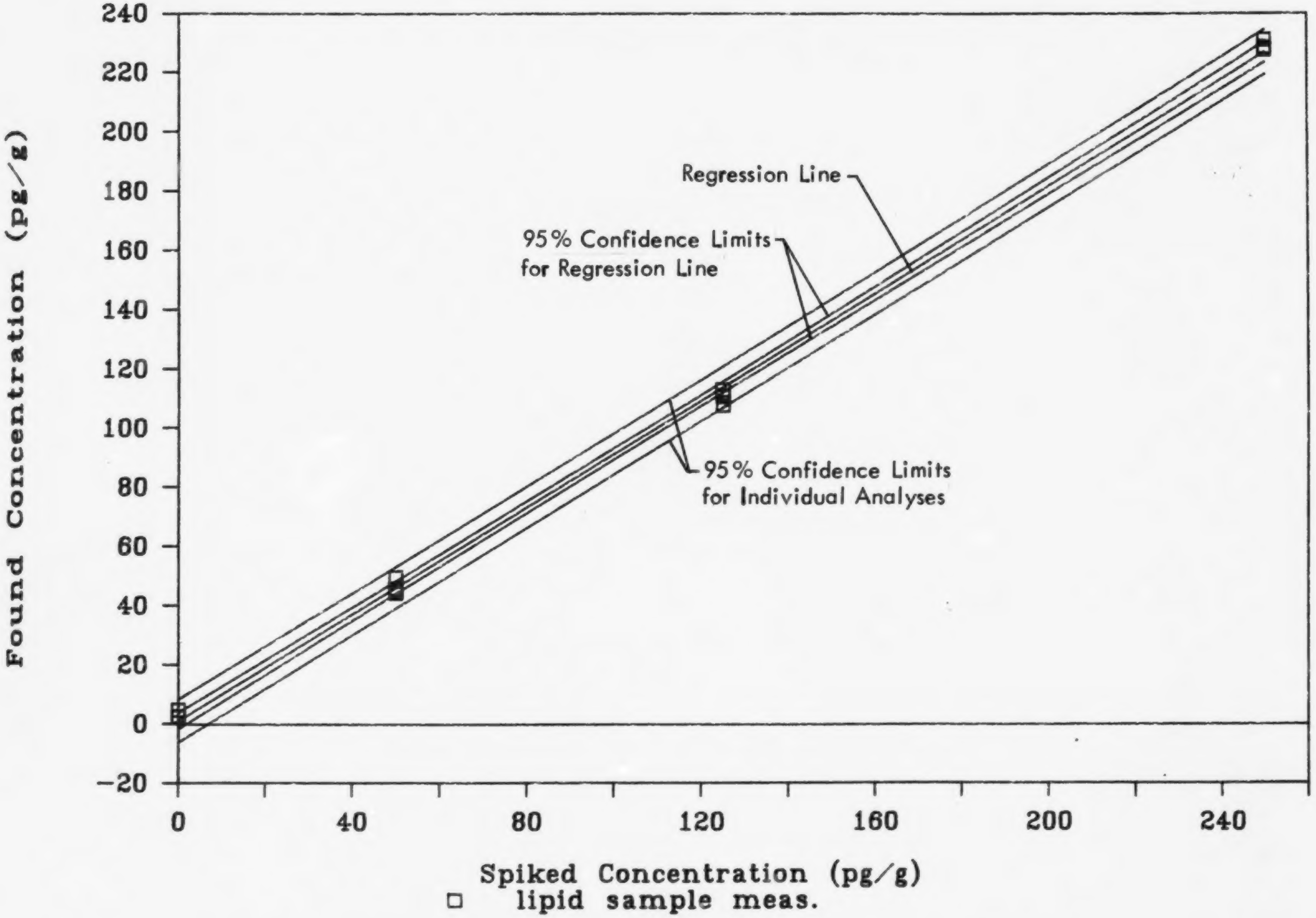

Figure 23. Measured concentrations versus concentrations of OCDF spiked into the homogenized human adipose lipid matrix.

The slopes of the calculated regression lines from the data points in each of the 14 analyses can be used as an indication of the accuracy of the analytical method for the 17 target analytes. Figure 24 is a plot of the slope of regression lines versus the 17 individual compounds. Table 16 provides a key to specific compounds associated with a number on the x-axis of this plot. The plot presents the estimated slope from each least squares regression line as well as the upper and lower 95% confidence limits for the slope. The slope of the regression line can be interpreted as a measure of accuracy with a value of 1.00 equivalent to 100% agreement of the measured concentration with the theoretical values (background plus spike level). The plot of the 95% confidence limits presents some confirmation on the precision of measurements across the four spike levels. These confidence bounds can also be used to determine whether the accuracy of the measurements (slope of regression line) is significantly different from 100% (or 1.00). If the vertical line connecting the lower and upper 95% confidence limits intersects with the horizontal line at 1, then the accuracy of the method (as determined from the regression line) is not significantly different from 100% (slope = 1.00). The results plotted in Figure 24 demonstrate that the method accuracies for 7 of the 17 analytes are not significantly different from 100%.

On the other hand, if the upper and lower confidence limits are both greater than or both less than 1.00, then the accuracy of the method is significantly different from 100%. The data presented graphically in Figure 24 indicate that some positive bias (greater than 100%) is associated with the method accuracies for 9 of the 17 analytes while the measurements for a single analyte (OCDF) result in a slightly negative (less than 100%) bias.

Table 16 provides a key to the compound identification in Figure 24 and tabulates the slope of the regression lines and the upper and lower 95% confidence limits for each of the 2,3,7,8-substituted PCDD and PCDF analytes. As noted from Table 16, method accuracy (as defined by the regression line slope) ranges from 90% for OCDF up to 121% for 1,2,3,7,8,9-HxCDF. The accuracies for all other measurements fall within a range of 97 to 115%. The overall method accuracies meet the initial accuracy objective of 50-115% identified in the project quality assurance program plan. However, the predicted accuracy results for individual analysis as defined by the 95% upper confidence limits indicate that this range should be adjusted to 50-130%.

The bias in the accuracy of the measurements may be a result of slight differences in the concentration calibration standards and the internal quantitation standard and native PCDD and PCDF spiking solutions. As a preliminary check on these differences, solutions of the low level and of the high level native spike combined with the internal quantitation standards were analyzed. The results of these analyses are provided in Table 17. Accuracy was calculated as measured/spiked x 100.

The results of these analyses suggest that bias observed in overall method accuracy is attributed to the differences in the spiking solutions versus the calibration standards. For instance, the four HxCDF isomers demonstrated a consistent positive bias to method accuracy based on the least squares regression analysis. The analysis of the spiking solutions, submitted as samples, also indicates a definite positive bias for the same four HxCDF isomers.

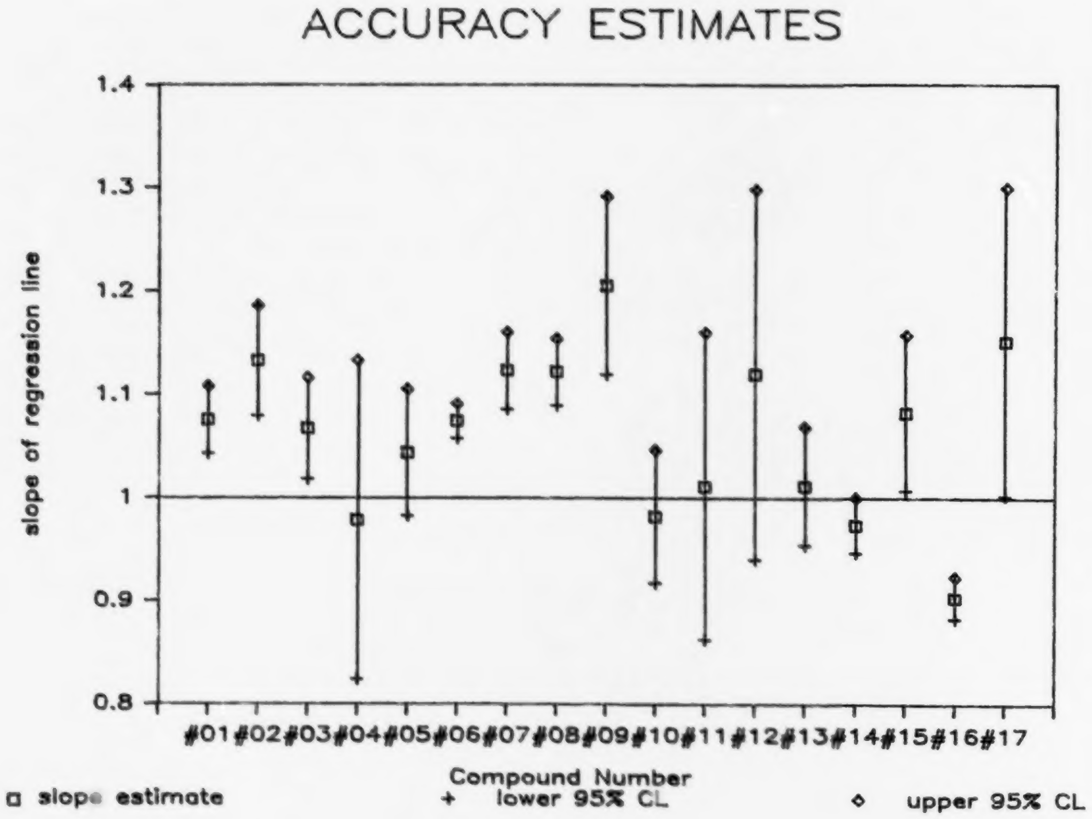

Figure 24. Method accuracy estimates as determined from the slopes of the least squares regression lines for the 17 target PCDD and PCDF analytes. Refer to Table 16 for the key to compound number.

Table 16. Regression Line Slopes with 95% Confidence Limits

Compound no.	Compound	Slope	Significantly different from 1.00?	Lower 95% confidence limit	Upper 95% confidence limit
01	2,3,7,8-TCDF	1.08	yes	1.04	1.11
02	2,3,7,8-TCDD	1.13	yes	1.08	1.19
03	1,2,3,7,8-PeCDF	1.07	yes	1.02	1.12
04	2,3,4,7,8-PeCDF	0.98	no	0.82	1.13
05	1,2,3,7,8-PeCDD	1.04	no	0.98	1.11
06	1,2,3,4,7,8-HxCDF	1.07	yes	1.06	1.09
07	1,2,3,6,7,8-HxCDF	1.12	yes	1.09	1.16
08	2,3,4,6,7,8-HxCDF	1.12	yes	1.09	1.15
09	1,2,3,7,8,9-HxCDF	1.21	yes	1.12	1.29
10	1,2,3,4,7,8-HxCDD	0.98	no	0.92	1.05
11	1,2,3,6,7,8-HxCDD	1.01	no	0.86	1.16
12	1,2,3,7,8,8-HxCDD	1.12	no	0.94	1.30
13	1,2,3,4,6,7,8-HpCDF	1.01	no	0.95	1.07
14	1,2,3,4,7,8,9-HpCDF	0.97	no	0.95	1.00
15	1,2,3,4,6,7,8-HpCDD	1.08	yes	1.01	1.16
16	OCDF	0.90	yes	0.88	0.92
17	OCDD	1.15	yes	1.00	1.30

Table 17. Results of the Analysis of the Low and High Level Native Spike Solutions

Compound	Low level spike Spike concentration (pg/μL)	Low level spike Measured concentration (pg/μL)	Low level spike Accuracy (%)	High level spike Spike concentration (pg/μL)	High level spike Measured concentration (pg/μL)	High level spike Accuracy (%)
2,3,7,8-TCDF	10	13	130	50	54	108
2,3,7,8-TCDD	10	12	120	50	57	114
1,2,3,7,8-PeCDF	10	11	110	50	52	104
2,3,4,7,8-PeCDF	10	10	100	50	49	98
1,2,3,7,8-PeCDD	10	12	120	50	53	106
1,2,3,4,7,8-HxCDF	25	27	108	125	134	107
1,2,3,6,7,8-HxCDF	25	32	128	125	137	110
2,3,4,6,7,8-HxCDF	25	35	140	125	152	122
1,2,3,7,8,9-HxCDF	25	40	160	125	185	148
1,2,3,4,7,8-HxCDD	25	27	108	125	123	98
1,2,3,6,7,8-HxCDD	25	24	96	125	132	106
1,2,3,7,8,9-HxCDD	25	39	156	125	148	118
1,2,3,4,6,7,8-HpCDF	25	22	88	125	116	93
1,2,3,4,7,8,9-HpCDF	25	25	100	125	121	97
1,2,3,4,6,7,8-HpCDD	25	26	104	125	130	104
OCDF	50	44	88	250	247	99
OCDD	50	48	96	250	250	100

Similar trends are noted for other compounds in Table 17 compared to the data presented in Figure 24 and Table 16.

The limited number of analyses of the spiking solutions does not provide an adequate comparison with the sample data to confirm the bias. However, it is recommended that at least triplicate measurements of the spiking solutions at each fortification level should be analyzed at the outset of the actual NHATS sample analysis program. This will be necessary to account for any biases that will be observed from the determination of PCDD and PCDF residue levels in spiked QC samples. It should be noted that additional homogenized spiked samples will be prepared prior to initiation of the NHATS sample analyses.

1. Recovery of Internal Quantitation Standards

The absolute recoveries for the carbon-13 labeled internal quantitation standards were determined for each sample by comparing the responses to the internal recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD. The average recoveries of the compounds in Tables 10 to 15 range from 52.1% for $^{13}C_{12}$-2,3,7,8-TCDD up to 88.9% for $^{13}C_{12}$-OCDD. The results for the absolute recoveries compared to the overall method accuracy for each compound indicate the importance of the internal standard quantitation technique for analysis of the PCDDs and PCDFs in human adipose.

2. Estimation of Background Levels of PCDDs and PCDFs

The estimated background levels of the various PCDD and PCDF congeners were determined as the intercept obtained from the least squares linear regression analyses. Table 18 provides a comparison of the average measured values for the unspiked matrix and the background concentration estimates from linear regression analysis of the data. In general, the measured and estimated background levels are in good agreement. However, several analytes with concentrations of less than 5 pg/g, particularly OCDF, demonstrate some disagreement in the measured versus estimated concentrations. This apparently arises from the fact that the first spike level is significantly greater than the actual background concentration. In the case of OCDF, the first spike level estimated by linear regression was 50 pg/g compared to an average measured value of 3.2 pg/g. In order to provide a better estimate of the background level based on the linear regression analysis, additional spike levels between 5 and 50 pg/g would be required. Table 18 also provides the upper and lower 95% confidence limits for the background levels estimated by the linear regression analysis of the data. These estimated background levels and confidence limits can be viewed as the intersections of the regression line and its upper and lower 95% confidence bounds, respectively, with the y-axis (measured or found concentration). These values will be used as the initial data points for developing control charts of the unspiked lipid matrix which will be analyzed with each batch of samples throughout the EPA/VA study.

Table 18. Background Level Estimates with 95% Confidence Limits

Compound no.	Compound	Measured background level (pg/g)[a]	Estimated[b] background level (pg/g)	Level significantly different from zero?	Lower 95% confidence limit (pg/g)	Upper 95% confidence limit (pg/g)
01	2,3,7,8-TCDF	ND (4.1)[c]	ND (3.6)	yes	2.7	4.5
02	2,3,7,8-TCDD	11.5	11.8	yes	10.4	13.3
03	1,2,3,7,8-PeCDF	ND (0.9)	ND (1.1)	no	-0.2	2.5
04	2,3,4,7,8-PeCDF	20.5	22.2	yes	17.9	26.4
05	1,2,3,7,8-PeCDD	19.4	19.8	yes	18.2	21.5
06	1,2,3,4,7,8-HxCDF	22.2	22.4	yes	21.3	23.5
07	1,2,3,6,7,8-HxCDF	12.4	11.2	yes	8.6	13.7
08	2,3,4,6,7,8-HxCDF	4.4	4.3	yes	2.1	6.5
09	1,2,3,7,8,9-HxCDF	ND (0.7)	ND (2.3)	no	-3.6	8.2
10	1,2,3,4,7,8-HxCDD	21.5	24.9	yes	20.5	29.3
11	1,2,3,6,7,8-HxCDD	157.75	159.4	yes	149.3	169.5
12	1,2,3,7,8,9-HxCDD	23.2	26.5	yes	14.3	38.7
13	1,2,3,4,6,7,8-HpCDF	28.9	25.7	yes	21.9	29.6
14	1,2,3,4,7,8,9-HpCDF	ND (1.1)	ND (0.3)	no	-2.1	1.5
15	1,2,3,4,6,7,8-HpCDD	213.7	214.0	yes	208.9	219.1
16	OCDF	3.2	ND (1.0)	no	-1.7	3.8
17	OCDD	806.1	799.7	yes	779.4	820.0

[a] The measured background levels are the averages of the triplicate analyses of the unspiked matrix.
[b] The estimated background levels were derived from the linear regression analysis of data.
[c] ND = not detected. The value in parentheses reflects the estimated method detection limit.

3. Day-to-Day HRGC/MS Analysis Precision

In addition to the analysis of the replicate spiked samples, four extracts were analyzed by HRGC/MS on two different dates. The results of the duplicate HRGC/MS analyses of these four samples for the 17 target compounds are presented in Tables 19 to 21. Concentration values from the second analysis date were included in the statistical analysis of data presented earlier in this section.

Table 19. Day-to-Day Precision of Analysis of Specific Sample Extracts for Tetra- and Pentachloro PCDF and PCDD

Analysis date	Spike level (pg/g)	2,3,7,8-TCDF (pg/g)	2,3,7,8-TCDD (pg/g)	1,2,3,7,8-PeCDF (pg/g)	2,3,4,7,8-PeCDF (pg/g)	1,2,3,7,8-PeCDD (pg/g)
4/22/86	0	ND (3.0)[a]	10	ND (0.84)	24	18
4/28/86	0	ND (4.1)	11	ND (1.12)	21	20
RPD (%)[b]		29	10	29	13	11
4/22/86	0	ND (3.1)	10	ND (0.78)	23	17
4/28/86	0	ND (4.1)	11	ND (0.75)	22	20
RPD (%)		28	10	4	4	16
4/22/86	0	ND (3.3)	10	ND (0.81)	16	17
4/28/86	0	ND (4.0)	13	ND (0.75)	19	18
RPD (%)		19	26	8	17	6
4/22/86	10	12	21	11	26	27
4/28/86	10	14	23	12	28	19
RPD (%)		18	9	8	7	35

[a] ND = not detected. Value in parentheses is the estimated limit of detection.
[b] Relative percent difference. Calculated as the difference of the two values divided by the mean of the two values times 100%.

Table 20. Day-to-Day Precision of Analysis of Specific Sample Extracts for Hexa- and Heptachloro PCDF and PCDD

Analysis date	Spike level (pg/g)	1,2,3,4,7,8-HxCDF (pg/g)	1,2,3,6,7,8-HxCDF (pg/g)	2,3,4,6,7,8-HxCDF (pg/g)	1,2,3,7,8,9-HxCDF (pg/g)	1,2,3,4,7,8-HxCDD (pg/g)	1,2,3,6,7,8-HxCDD (pg/g)	1,2,3,7,8,9-HxCDD (pg/g)	1,2,3,4,6,7,8-HpCDF (pg/g)	1,2,3,4,7,8,9-HpCDF (pg/g)	1,2,3,4,6,7,8-HpCDD (pg/g)
4/22/86	0	21	12	4.2	ND (0.33)[a]	20	149	16	28	ND (0.83)	210
4/28/86	0	22	12	4.9	ND (0.51)	22	170	19	31	ND (0.36)	216
RPD (%)[b]		5	0	15	43	10	13	17	10	79	3
4/22/86	0	22	12	4.0	ND (0.32)	21	150	18	25	ND (1.09)	207
4/28/86	0	22	12	4.2	ND (0.74)	23	178	26	27	ND (1.14)	211
RPD (%)		0	0	5	79	9	17	36	8	4	2
4/22/86	0	20	12	3.9	ND (0.41)	21	176	18	27	ND (1.06)	223
4/28/86	0	23	13	4.3	ND (0.86)	20	171	25	29	ND (0.28)	216
RPD (%)		14	8	10	71	5	3	33	7	116	3
4/22/86	25	44	36	28	30	43	171	46	46	23	235
4/28/86	25	47	37	32	35	49	181	63	49	25	241
RPD (%)		6	3	13	15	13	6	31	6	8	3

[a] ND = not detected. The value in parentheses is the estimated limit of detection.
[b] Relative percent difference. Calculated as the difference of the two values divided by the mean of the two values times 100%.

Table 21. Day-to-Day Precision of Analysis of Specific Sample Extracts for OCDF and OCDD

Analysis date	Spike level (pg/g)	OCDF concentration (pg/g)	OCDD concentration (pg/g)
4/22/86	0	4.9	811
4/28/86	0	3.5	810
RPD (%)[a]		33	0.1
4/22/86	0	2.2	819
4/28/86	0	2.3	836
RPD (%)		4	2
4/22/86	0	1.9	788
4/28/86	0	2.6	784
RPD (%)		31	1
4/22/86	50	43	834
4/28/86	50	44	848
RPD (%)		2	2

[a] Relative percent difference. Calculated as the difference of the two values divided by the mean of the two values times 100%.

VI. QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

As discussed in the experimental section of this report, the QA/QC activities included the analysis of a multipoint calibration curve, daily verification of relative response factors for each analyte, analysis of a method blank and reagent blanks along with the samples, and determining the absolute recoveries of each of the internal quantitation standards for every sample. Each of these QA/QC activities is discussed below.

A. Initial Calibration

At the outset of sample analysis activity, six calibration concentration standards containing each of the target PCDDs and PCDFs at varying levels and constant concentrations of the internal quantitation and recovery standards were analyzed in triplicate. The relative response factors (RRF) for each native compound and internal quantitation standard were determined for each standard analysis. An average RRF and relative percent standard deviation (RSD) were determined for each concentration level. The average RRF values from each of the six concentration calibration standards were then used to calculate a grand mean RRF value for each compound in the calibration solution. Table 22 presents a summary of the grand mean RRF values for each component in the standards. As noted from Table 22, the average RRF values for native PCDDs and PCDFs generally varied by less than ± 10% (RSD) with the exception of the pentachloro congeners. These results fall well within the criteria established in the draft quality assurance program plan which required the variability of RRF values for the tetrachloro homologs to be within ± 20% (RSD) while the RRF criterion on all other compounds was set at ± 30% (RDS).

The variability of the RRF values for the internal quantitation standards, on the other hand, was noted to increase with the degree of chlorination. This is a result of the measurement of all internal quantitation standards versus the single internal recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD. A second internal recovery standard, $^{37}Cl_4$-1,2,3,4,6,7,8-HpCDD, was evaluated. However, problems resulting from contribution of native HpCDD to the characteristic ions of this internal standard resulted in variabilities in the RRF value up to 50%. Hence, this internal standard was not used for any calculations. It is anticipated that an additional internal recovery standard, such as $^{13}C_{12}$-1,2,3,4,7,8-HxCDD, will reduce the variability in the RRF values of the higher chlorinated internal quantitation standards. This compound will be incorporated into the method if available.

The sensitivity of the Kratos MS-50TC to the tetra- through octachloro PCDDs and PCDFs was demonstrated through the triplicate analysis of the low level standard (CS-8, Table 2) that ranged in concentration from 1 pg/µL for the tetra- and pentachloro congeners up to 5 pg/µL for the octachloro congeners. These solution concentration values of 1 pg/µL and 5 pg/µL correspond to residue levels in tissue of 1 pg/g and 5 pg/g, respectively. Table 22 provides an indication of the observed signal-to-noise ratio for each of the native PCDD and PCDF congeners. These data demonstrate that the low level standard is well above the instrument detection limit, which is defined as the amount of a particular compound necessary to give a signal 2.5 times the background signal to noise for each of the characteristic ions while meeting the qualitative criteria for ion ratios.

Table 22. Relative Response Factors (Grand Means) Determined from Multipoint Concentration Calibration Standards

Compound	$\overline{RRF}$[a]	RSD (%)	RRF control limits[b]	Signal-to-noise ratio for low level standard[c]	Calibration range (pg/μL)
2,3,7,8-TCDF	1.00	5.7	0.80-1.20	12	1-100
2,3,7,8-TCDD	0.80	6.2	0.64-0.96	6.5	1-100
1,2,3,7,8-PeCDF	0.98	5.2	0.68-1.28	11	1-100
2,3,4,7,8-PeCDF	1.06	10.1	0.74-1.38	8.9	1-100
1,2,3,7,8-PeCDD	1.33	11.3	0.93-1.73	5.7	1-100
1,2,3,4,7,8-HxCDF	0.94	3.1	0.66-1.22	32	2.5-250
1,2,3,6,7,8-HxCDF	0.93	2.4	0.65-1.21	30	2.5-250
2,3,4,6,7,8-HxCDF	0.86	2.7	0.60-1.12	29	2.5-250
1,2,3,7,8,9-HxCDF	0.86	6.9	0.60-1.12	17	2.5-250
1,2,3,4,7,8-HxCDD	1.31	4.9	0.92-1.70	13	2.5-250
1,2,3,6,7,8-HxCDD	1.44	3.0	1.01-1.87	14	2.5-250
1,2,3,7,8,9-HxCDD	1.61	1.0	1.13-2.09	14	2.5-250
1,2,3,4,6,7,8-HpCDF	2.33	4.0	1.63-3.03	35	2.5-250
1,2,3,4,7,8,9-HpCDF	1.89	3.6	1.32-2.46	26	2.5-250
1,2,3,4,6,7,8-HpCDD	1.19	4.7	0.83-1.55	13	2.5-250
OCDF	1.38	3.3	0.97-1.79	31	5-500
OCDD	1.04	2.5	0.73-1.35	21	5-500
$^{13}C_{12}$-1,2,3,4-TCDD[d]	1.00	-	-	-	50
$^{13}C_{12}$-2,3,7,8-TCDF	1.98	7.6	1.58-2.38	-	50
$^{13}C_{12}$-2,3,7,8-TCDD	1.73	4.7	1.38-2.08	-	50
$^{13}C_{12}$-1,2,3,7,8-PeCDF	1.36	4.5	0.95-1.77	-	50
$^{13}C_{12}$-1,2,3,7,8-PeCDD	0.70	7.7	0.49-0.91	-	50
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	1.28	15.8	0.90-1.66	-	125
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	0.41	19.6	0.29-0.53	-	125
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	0.33	25.8	0.23-0.43	-	125
$^{37}Cl_4$-1,2,3,4,6,7,8-HpCDD[d]	0.12	51.8	-	-	125
$^{13}C_{12}$-OCDD	0.24	28.0	0.17-0.31	-	250

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[a] $\overline{RRF}$ = grand mean RRF.

[b] RRF control limits designate the acceptable range of values based on the criteria for ± 20% of the $\overline{RRF}$ for 2,3,7,8-TCDD and 2,3,7,8-TCDF and ± 30% of the $\overline{RRF}$ for all other PCDD and PCDF compounds.

[c] Value for signal-to-noise ratio based on observed response for the major characteristic ion for each native PCDD or PCDF congener (Data File 8501D17X02).

[d] Internal recovery standard.

B. Daily Verification of Response Factors

Before proceeding with analysis of samples, the analyst was required to verify the existing response factor calibration through the analysis of a calibration standard (CS-7, Table 2). Criteria for proceeding with sample analysis required that the measured RRF value for 2,3,7,8-TCDD and 2,3,7,8-TCDF were within ± 20% (and all other congeners within ± 30%) of the mean RRF established from the calibration curve. This standard was also analyzed at the end of each working day to demonstrate that the calibration had been maintained. All RRF values were tabulated to generate RRF control charts for each specific PCDD and PCDF congener.

Figures 25 through 34 are plots (control charts) of the RRF values established for the 17 individual target analytes. The RRF data are plotted versus time of analysis. These plots contain 28 individual data points, 18 of which were generated for triplicate analysis of 6 concentration calibration solutions from initial calibration and 10 analyses of solution CS-7 (Table 2) injected over the 5 days for which actual samples were analyzed. The upper and lower boundaries (dashed lines) represent a relative standard deviation of approximately ± 10% with the exception of the plot for 1,2,3,7,8-PeCDD, for which the boundaries are plotted as ± 20%.

It should be noted that the actual control limits as specified in the project QAPP were set at ± 20% for 2,3,7,8-TCDD and 2,3,7,8-TCDF and ± 30% for all other target analytes. Boundaries of ± 10% have been used in Figures 25 through 34 as a means to provide the reader with a better perspective in the actual distribution of the measured calibration points. The values for the acceptable ranges of each PCDD and PCDF compound based on the initial calibration are presented in Table 22. The data presented for the RRF values in Figures 25 through 34 are well within these established control limits. The average RRF values and corresponding standard deviations reported in each of these plots are calculated from the total 28 standard analyses.

C. Blanks

As specified in the quality assurance program plan, a laboratory method blank was prepared along with the 14 human adipose lipid samples. The method blank was taken through all procedures as if it were an actual sample, although no lipid matrix was introduced. The analysis of the method blank resulted in the data reported for each of the target analytes reported in Table 23. As noted in Table 23, 1,2,3,4,6,7,8-HpCDD and OCDD were detected at concentrations equivalent to 4.0 and 30 pg/g (equivalent to a 10-g lipid sample), respectively.

The contribution of these PCDDs were not subtracted from the observed responses for the spiked and unspiked samples. These background levels accounted for less than 2% of the 1,2,3,4,6,7,8-HpCDD and less than 4% of the OCDD measured in the unspiked lipid samples. In addition to these compounds, responses that correspond to the elution of two TCDD isomers (1,3,6,8- and 1,3,7,9-) and a PeCDD (isomer not determined) were detected in the method blank.

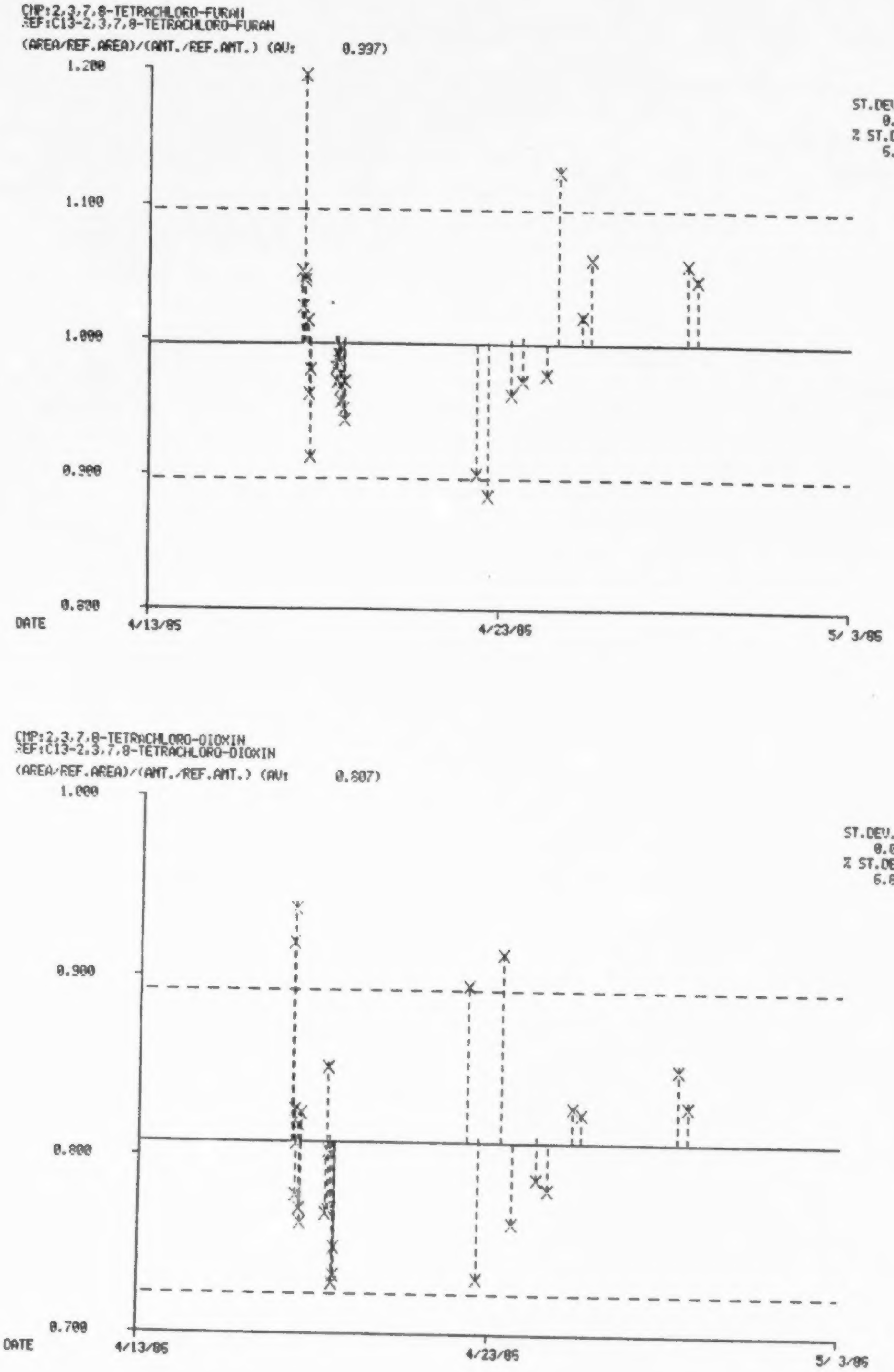

Figure 25. Control charts showing response factors by date for 2,3,7,8-TCDF and 2,3,7,8-TCDD. Dotted lines represent approximately ± 10% of the mean.

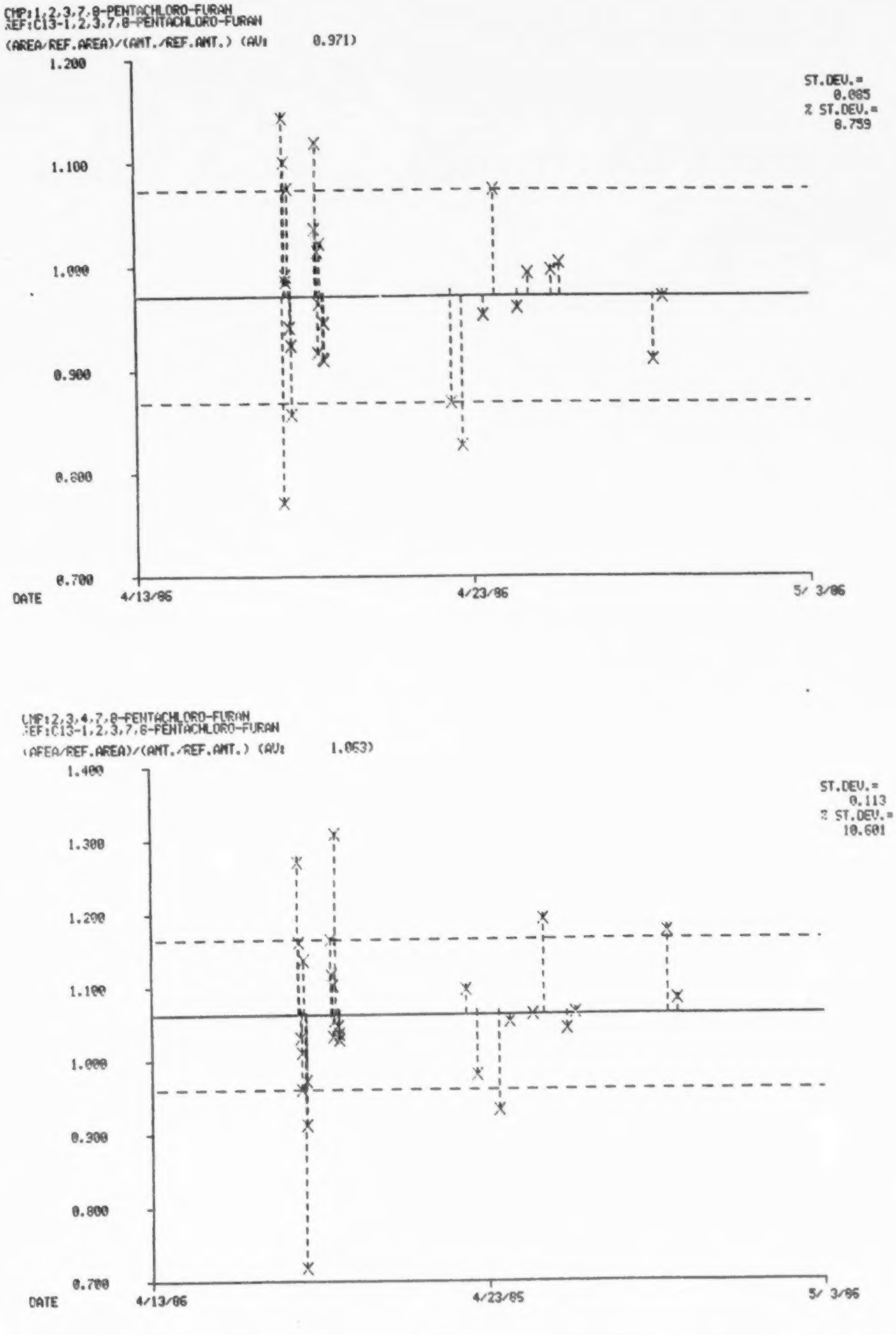

Figure 26. Control charts showing response factors by date for 1,2,3,7,8-PeCDF and 2,3,4,7,8-PeCDF. Dotted lines represent approximately ± 10% of the mean.

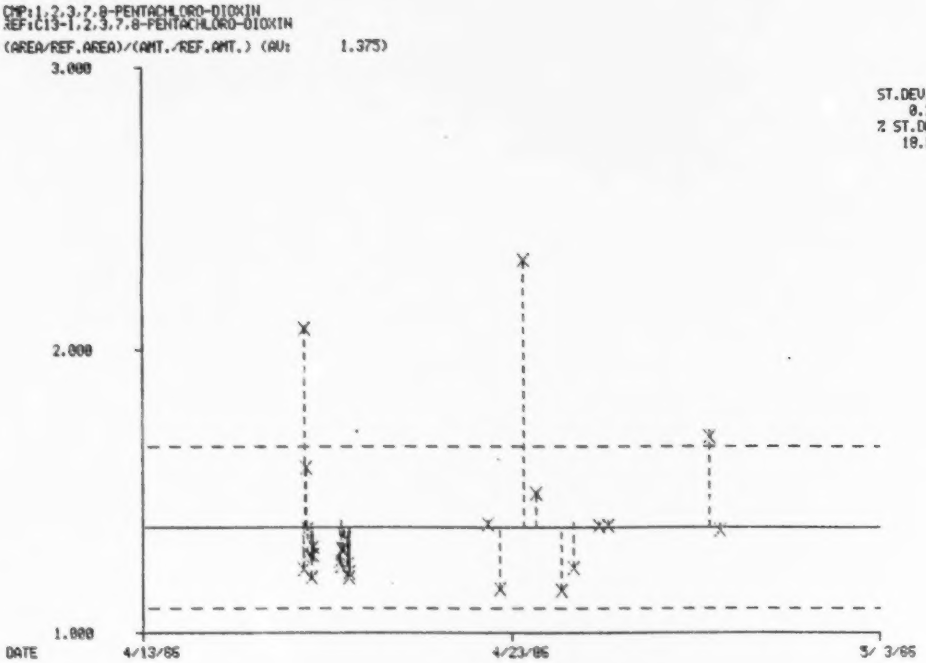

Figure 27. Control chart showing response factors by date for 1,2,3,7,8-PeCDD. Dotted lines represent approximately ± 20% of the mean.

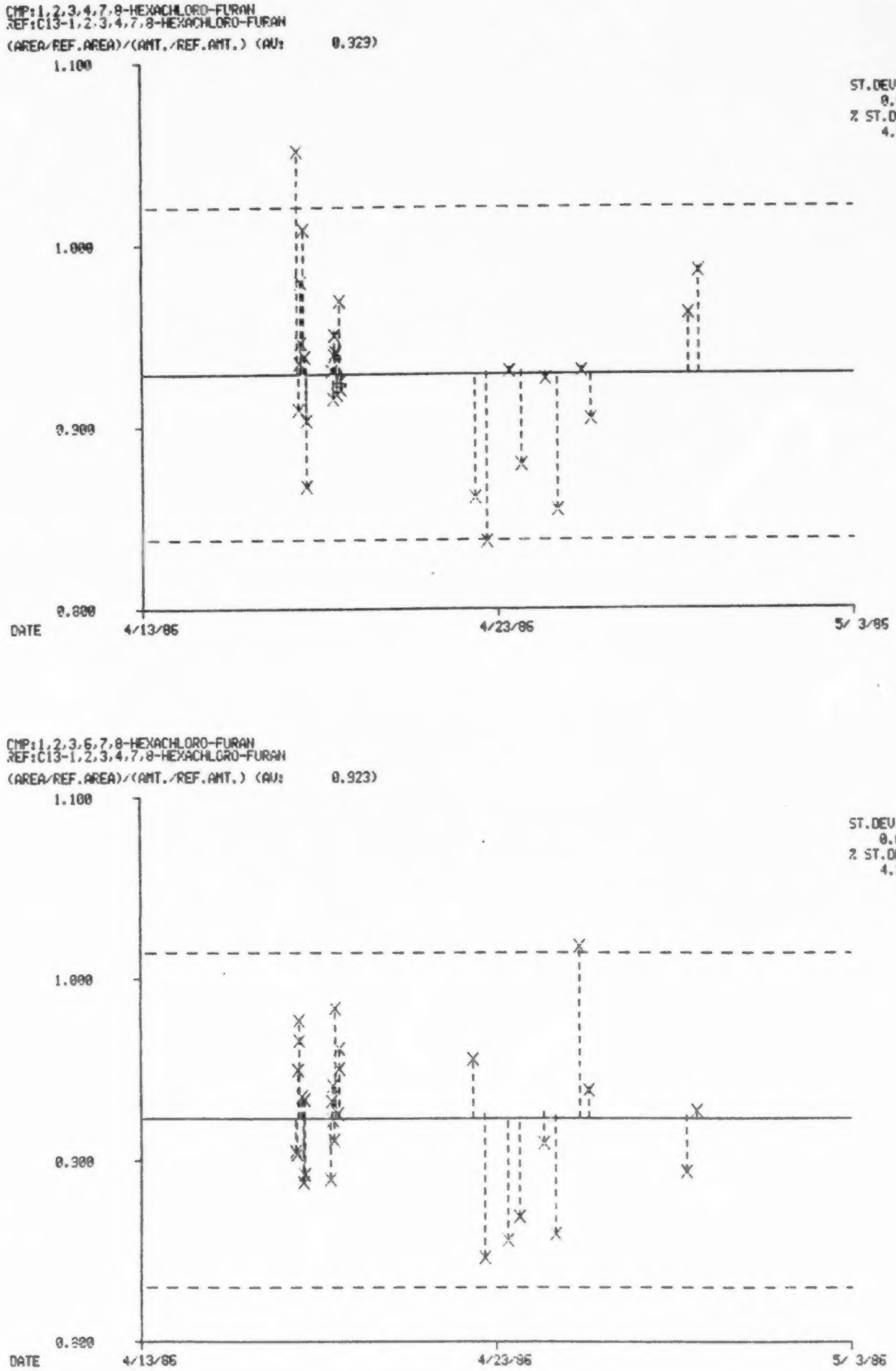

Figure 28. Control charts showing response factors by date for 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF. Dotted lines represent approximately ± 10% of the mean.

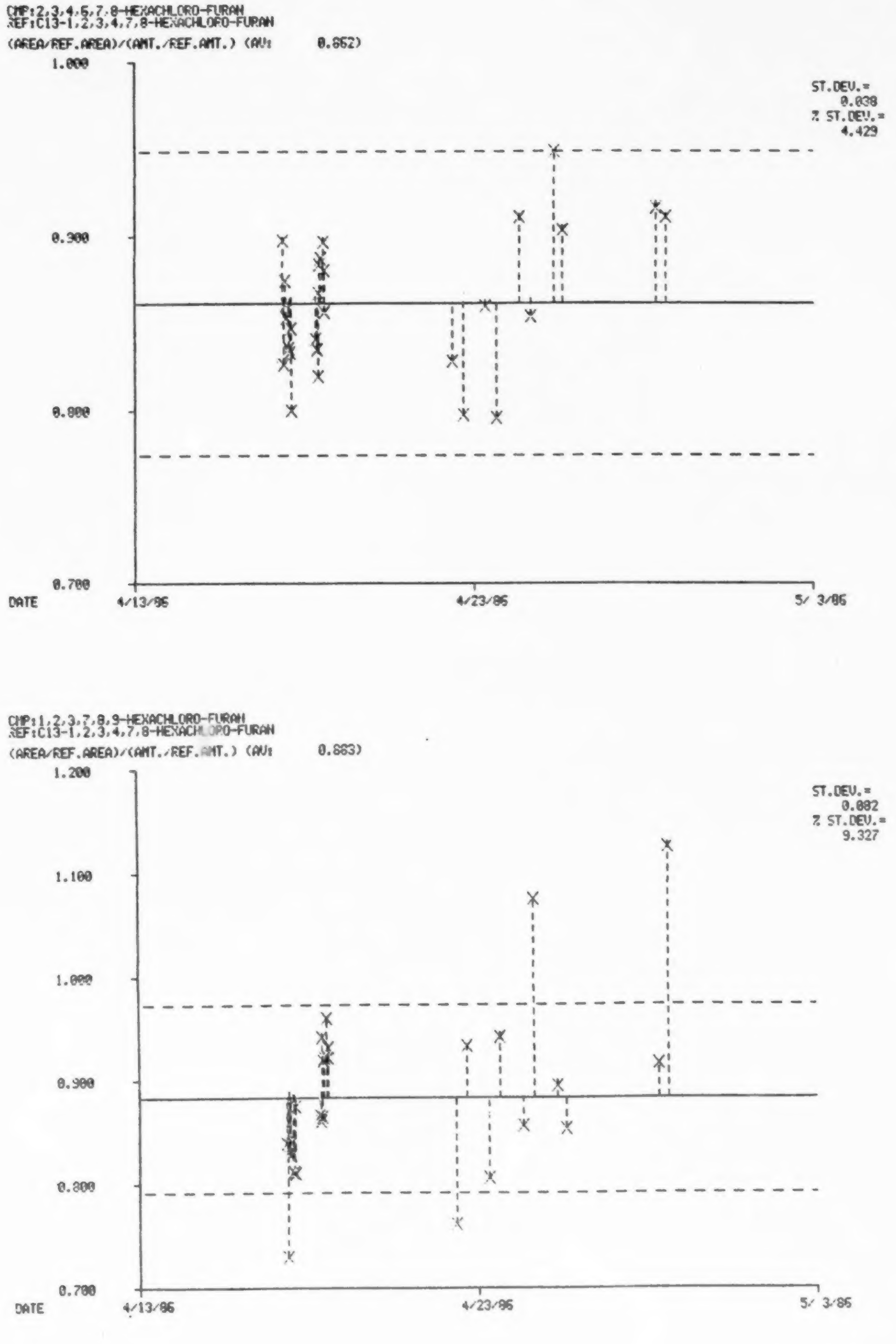

Figure 29. Control charts showing response factors by date for 2,3,4,6,7,8-HxCDF and 1,2,3,7,8,9-HxCDF. Dotted lines represent approximately ± 10% of the mean.

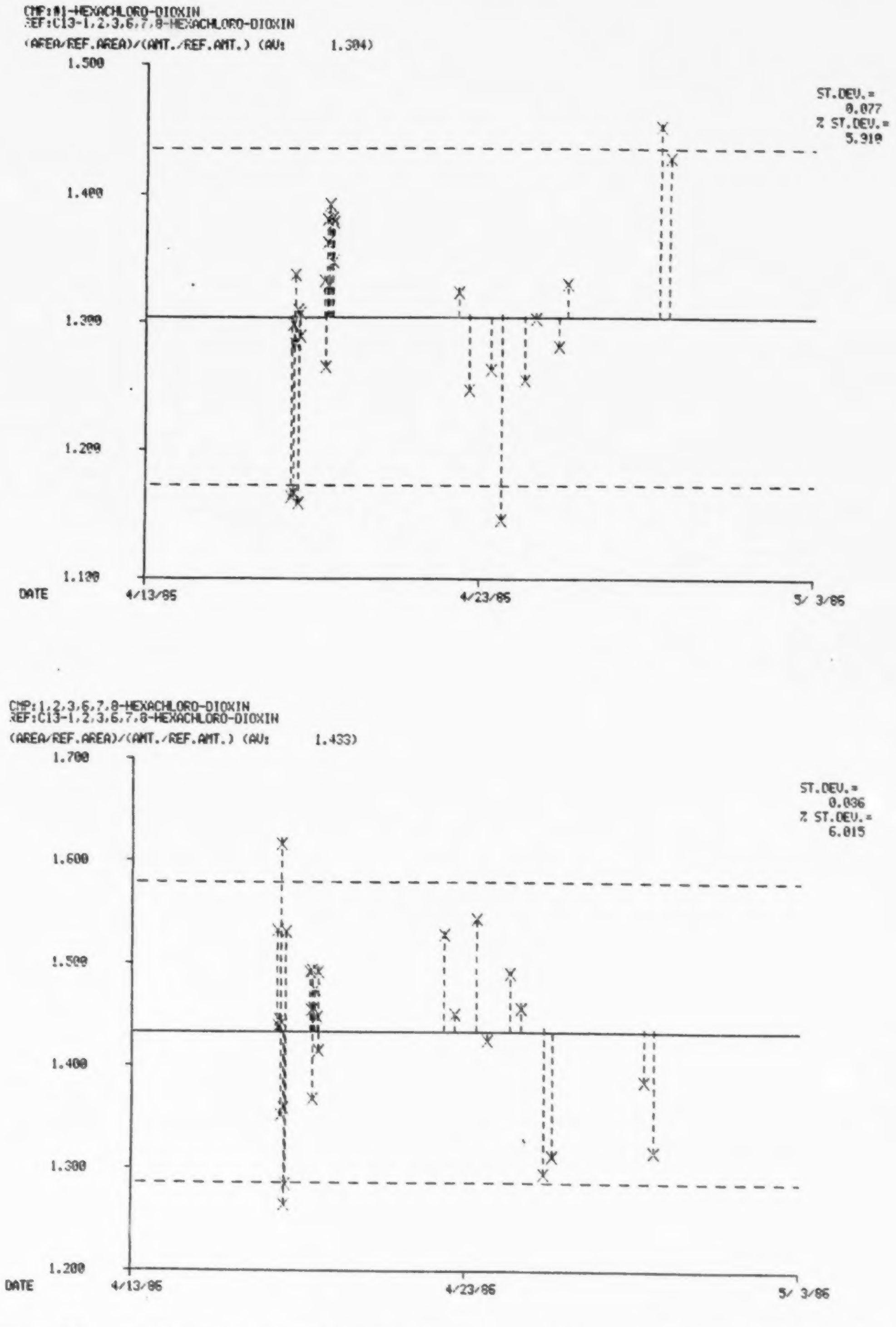

Figure 30. Control charts showing response factors by date for 1,2,3,4,7,8-HxCDD and 1,2,3,6,7,8-HxCDD. Dotted lines represent approximately ± 10% of the mean.

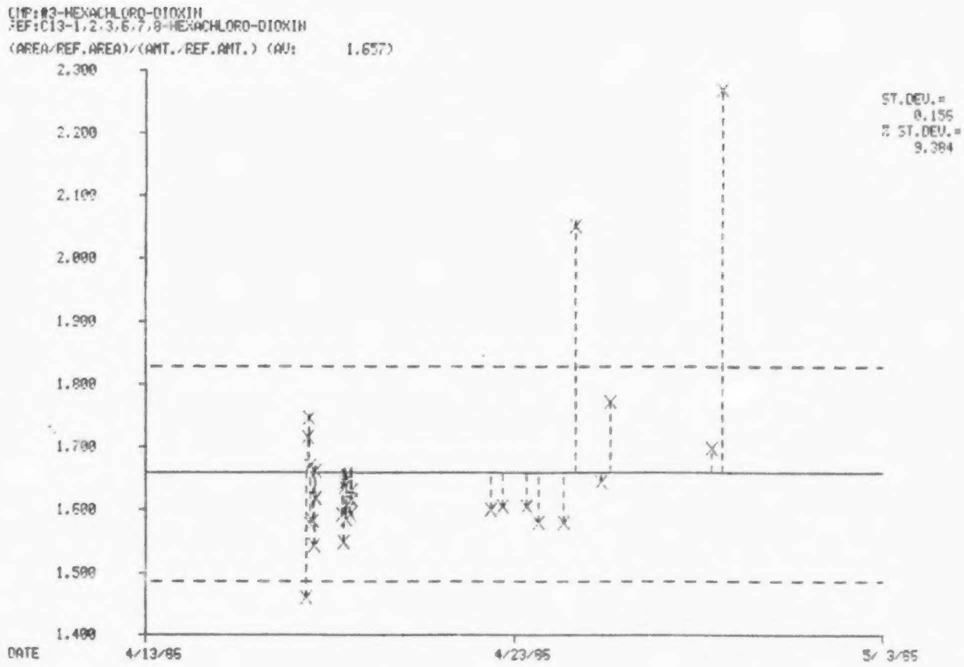

Figure 31. Control chart showing response factors by date for 1,2,3,7,8,9-HxCDD. Dotted lines represent approximately ± 10% of the mean.

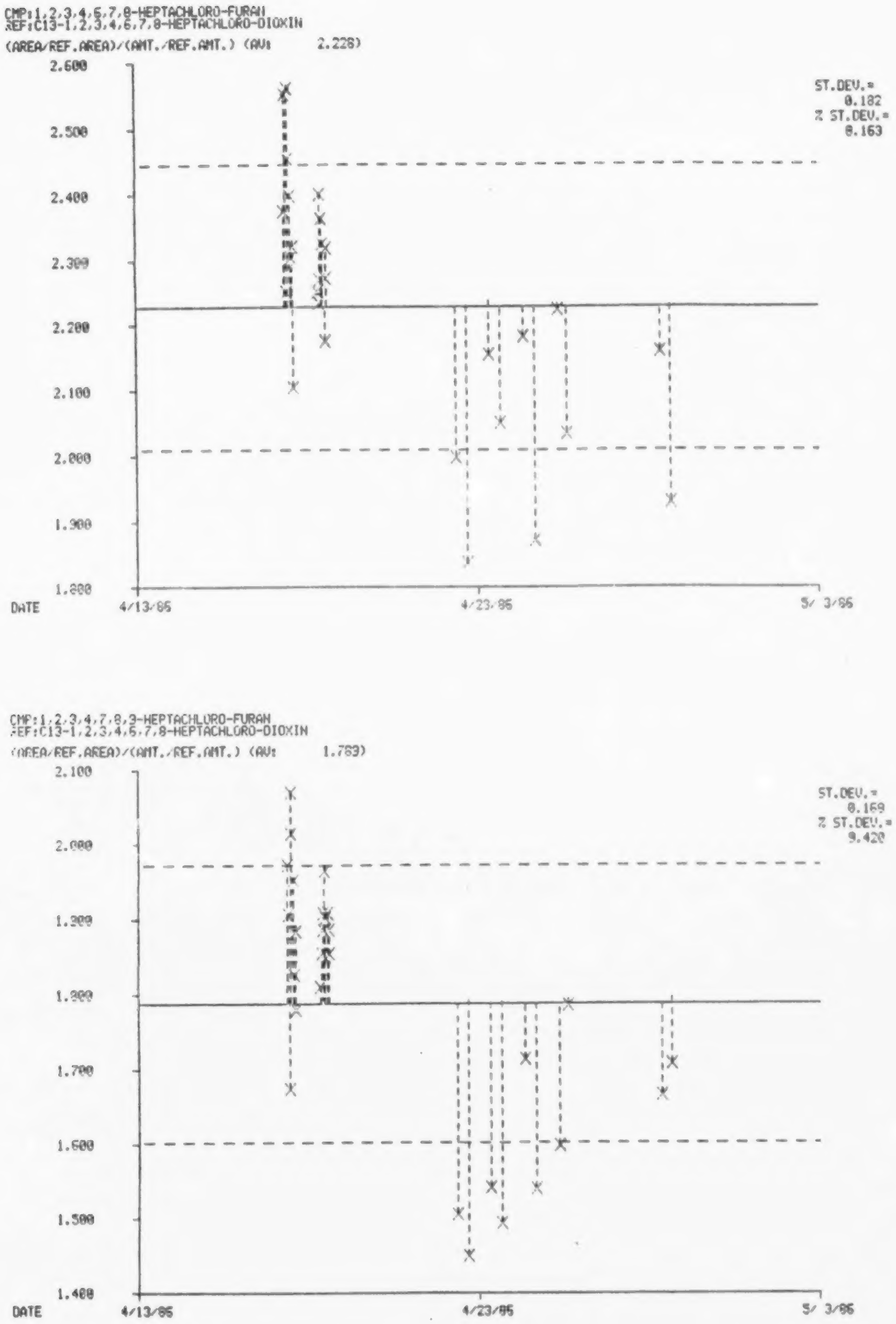

Figure 32. Control charts showing response factors by date for 1,2,3,4,6,7,8-HpCDF and 1,2,3,4,7,8,9-HpCDF. Dotted lines represent approximately ± 10% of the mean.

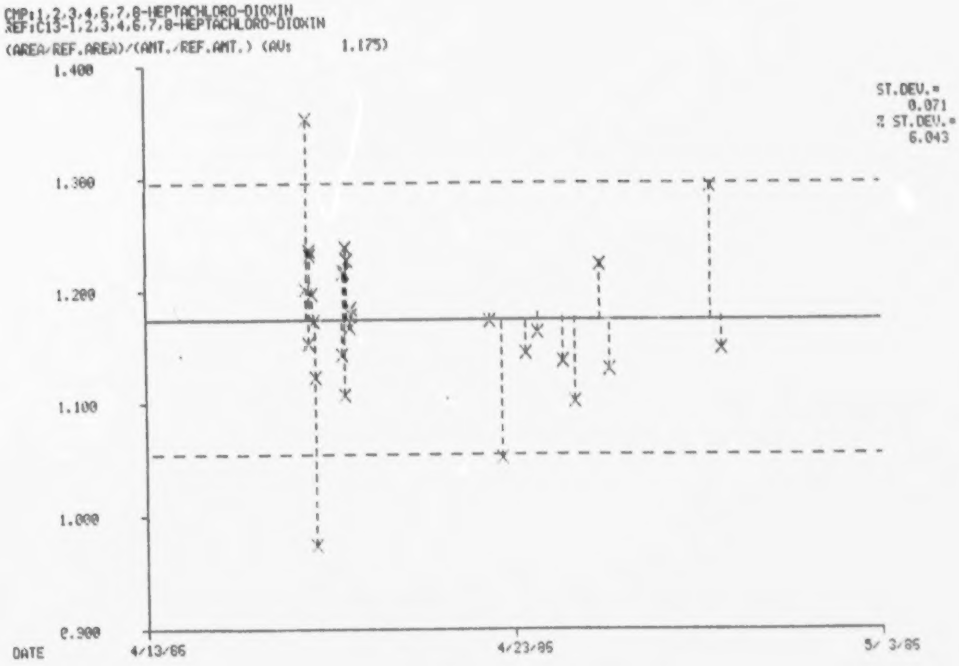

Figure 33. Control chart showing response factors by date for 1,2,3,4,6,7,8-HpCDD. Dotted lines represent approximately ± 10% of the mean.

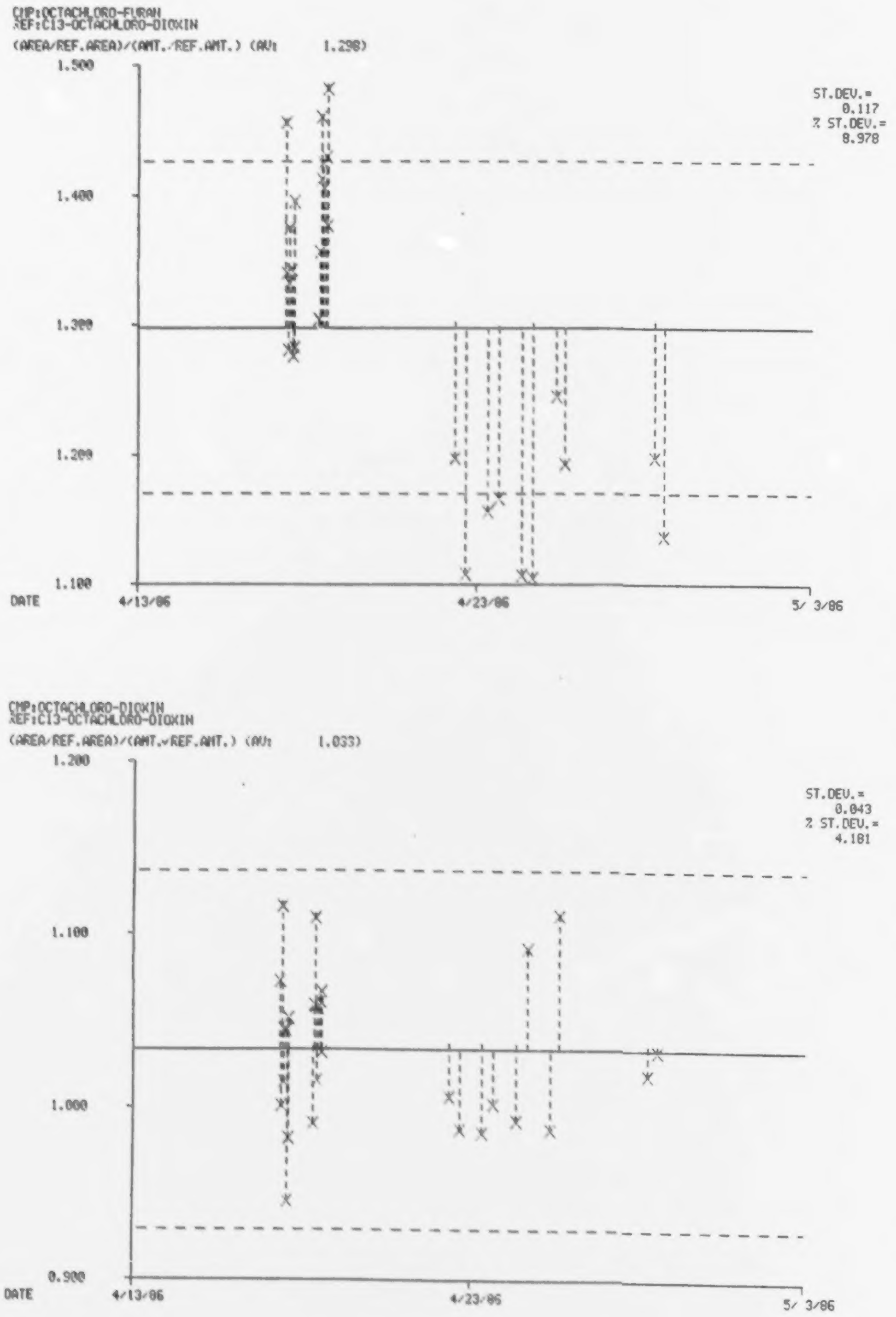

Figure 34. Control charts showing response factors by date for OCDF and OCDD. Dotted lines represent approximately ± 10% of the mean.

Table 23. Summary of Results from the Analysis of a Laboratory Method Blank

Compound[a]	Concentration[b] (pg/g)
2,3,7,8-TCDD	ND (0.50)
2,3,7,8-TCDF	ND (2.2)
1,2,3,7,8-PeCDF	ND (0.5)
2,3,4,7,8-PeCDF	ND (0.5)
1,2,3,7,8-PeCDD	ND (1.2)
1,2,3,4,7,8-HxCDF	ND (0.5)
1,2,3,6,7,8-HxCDF	ND (0.5)
2,3,4,6,7,8-HxCDF	ND (0.5)
1,2,3,7,8,9-HxCDF	ND (0.5)
1,2,3,4,7,8-HxCDD	ND (1.0)
1,2,3,6,7,8-HxCDD	ND (0.9)
1,2,3,7,8,9-HxCDD	ND (0.8)
1,2,3,4,6,7,8-HpCDF	ND (0.5)
1,2,3,4,7,8,9-HpCDF	ND (0.5)
1,2,3,4,6,7,8-HpCDD	4.0
OCDF	ND (0.5)
OCDD	30

[a] At least three other PCDD compounds were detected but not quantitated in the laboratory method blank. These included 1,3,6,8- and 1,3,7,9-TCDD and an unidentified PeCDD isomer.

[b] Concentration based on assumption of 10.0 g equivalent lipid sample. The background concentration of 1,2,3,4,6,7,8-HpCDD and OCDD were not subtracted from the measured concentration for the spiked and unspiked lipid matrix.

Further analysis of individual reagents used for preparation of the samples identified the activated acidic alumina as the source of the artifacts. Acidic alumina that had been cleaned by Soxhlet extraction but not activated at 190°C was analyzed, and the artifacts were not detected. This indicates that the artifacts are generated during activation of acidic alumina at elevated temperatures (190°C). Similar background problems from the same PCDD congeners have recently been reported by the Center for Disease Control.[17,18]

An experiment was designed to evaluate a procedure for cleaning the activated acidic alumina immediately prior to the fractionation of the sample extract. The acidic alumina (6.0 g) was packed in hexane. The packed column was eluted with 40 mL of methylene chloride/hexane (1:1) solution followed by 80 to 100 mL of hexane. The sample extract was added to the column and was eluted with 20 mL of hexane followed by 30 mL of 20% methylene chloride in hexane which was reserved for PCDD and PCDF analysis.

The carbon-14 radiolabeled 2,3,7,8-TCDD, 1,2,3,4,7,8-HxCDD, and OCDD were used to evaluate recovery of PCDDs from the cleaned alumina. Recoveries of the radiolabeled PCDDs from the activated acidic alumina precleaned by the procedure described above are detailed in Table 24. These data demonstrate that the selected PCDDs are quantitatively (greater than 90%) recovered from the precleaned acidic alumina. This procedure for cleanup of activated acidic alumina was not initiated for the analysis of the lipid samples described in this report. However, it has been integrated into the analytical protocol (Appendix A) for routine application with sample preparation activities.

D. Absolute Recoveries of the Internal Quantitation Standards

The absolute recoveries of the carbon-13 labeled internal quantitation standards were determined by comparing responses with the internal recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD, which was added during final concentration prior to HRGC/MS analysis. A summary of the average and range of recoveries of the 8 internal quantitation standards from the 14 human adipose lipid samples is provided in Table 25.

These data indicate that recoveries ranged from an average of 52.1% for $^{13}C_{12}$-2,3,7,8-TCDD up to 88.9% for $^{13}C_{12}$-OCDD. The average recoveries for the lower chlorinated internal standards were lower than the preliminary method studies with carbon-14 radiolabeled standards had indicated. This resulted in a closer evaluation of the final concentration step prior to mass spectrometry. The first extracts for the human adipose lipid extracts were concentrated with a nitrogen evaporation system equipped with a water bath at approximately 55°C. Final blowdown of the samples required addition of the internal recovery standard in 10 µL of tridecane as a keeper solution. However, it was noted at the elevated temperature final volumes from nitrogen evaporation were generally on the order of 2 to 5 µL. This required addition of another 10 µL of tridecane prior to HRGC/MS analysis.

In an effort to assess the effect of reducing the final volume of tridecane at elevated temperatures on absolute recoveries of the internal quantitation standards, an experiment using the radiolabeled TCDD, HxCDD, and OCDD standards was conducted.

Table 24. Recovery of Radiolabeled PCDDs from Precleaned Activated Alumina

Compound	Spike level (pg)	Recovery (%)
^{14}C-2,3,7,8-TCDD	100	92
	300	96
	300	97
^{14}C-1,2,3,4,7,8-HxCDD	1,000	103
	3,000	101
	3,000	100
^{14}C-OCDD	2,500	102
	7,500	99
	7,500	97

Table 25. Absolute Recoveries of the Internal Quantitation Standards from the Human Adipose Lipid Matrix[a]

Internal quantitation standard	Average recovery (%)	Standard deviation	Relative standard deviation (%)	Range of recovery (%)
$^{13}C_{12}$-2,3,7,8-TCDF	64.0	7.9	12.3	48-78
$^{13}C_{12}$-2,3,7,8-TCDD	52.1	5.0	9.6	43-62
$^{13}C_{12}$-1,2,3,7,8-PeCDF	76.1	8.9	11.7	62-90
$^{13}C_{12}$-1,2,3,7,8-PeCDD	57.1	3.5	6.1	51-64
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	59.4	5.0	8.4	52-70
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	63.6	5.3	8.4	57-77
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	76.3	10.3	13.6	61-99
$^{13}C_{12}$-OCDD	88.9	11.4	12.9	67-104

[a] Values based on 14 analyses of human adipose lipid samples.

Four solutions of the same spike level were prepared with each radiolabeled compound in 1 mL of toluene. Two of the spiked solutions were heated at 55-60°C and the solvent was reduced under a gentle stream of prepurified nitrogen. The toluene solution was concentrated to 100 µL, 500 µL of 1% toluene in methylene chloride was added, and the solution was concentrated to 200 µL. At this time 10 µL of the keeper tridecane was added and the solution was allowed to concentrate further. The remaining two solutions for each radiolabeled compound were taken through a similar solvent exchange and concentration procedure except the solution was allowed to concentrate at room temperature.

One of the most obvious results was the observation that solutions held at elevated temperatures could be reduced to dryness even when tridecane had been added as a keeper. On the other hand, solutions for which tridecane had been added but remained at room temperature could only be concentrated to a 10-µL final volume. The recoveries of the radiolabeled standards from each of the solutions in this study are presented in Table 26.

The results from this study indicate that the final concentration condition may have a pronounced effect on the absolute recoveries of the PCDDs and PCDFs, especially for the lower chlorinated congeners such as 2,3,7,8-TCDD. These conclusions are supported by an independent study in comparison of concentration techniques for 2,3,7,8-TCDD.[19] However, it should be noted that the approach to target analyte quantitation based on the internal standard method (isotope dilution for 8 of the 17 target analytes) is not affected by absolute recoveries as low as 50%. The procedure for final concentration in the analytical protocol (Appendix A) for the analysis of the NHATS samples for the EPA/VA study has been modified to specify room temperature conditions.

Table 26. Recovery of Carbon-14 Labeled 2,3,7,8-TCDD, 1,2,3,4,7,8-HxCDD, and OCDD as a Function of Final Concentration Conditions

Compound	Spike level (pg)	Concentration conditions[a]	Observed final volume	Observed recovery (%)
^{14}C-2,3,7,8-TCDD	300	55-60°C	1-2 μL	78
	300	55-60°C	dryness	54
	300	20°C	10 μL	98
	300	20°C	10 μL	93
^{14}C-1,2,3,4,7,8-HxCDD	3,000	55-60°C	1-2 μL	94
	3,000	55-60°C	5 μL	102
	3,000	20°C	10 μL	105
	3,000	20°C	10 μL	107
^{14}C-OCDD	7,500	55-60°C	1-2 μL	94
	7,500	55-60°C	2-3 μL	94
	7,500	20°C	10 μL	100
	7,500	20°C	10 μL	97

[a] Each solution was concentrated under a gentle stream of flowing nitrogen.

VII. GLOSSARY OF TERMS

Accuracy - A measurement of the bias of a system, which for this study, is based on the agreement of the 2,3,7,8 substituted PCDD and PCDF to an accepted reference standard.

Batch, sample - A sample batch consists of up to 10 human adipose tissue samples, one method blank, 2 internal quality control (QC) samples (spiked and unspiked), and an external performance audit sample (blind spike).

Blank, laboratory method - This blank is prepared in the laboratory through performing all analytical procedures except addition of a sample aliquot to the extraction vessel. A minimum of one laboratory method blank will be analyzed with each batch of samples.

Calibration standards (concentration calibration solutions) - Solutions containing known amounts of the native analytes (unlabeled 2,3,7,8-substituted PCDDs and PCDFs), the internal quantitation standards (carbon-13 labeled PCDDs and PCDFs), and the recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD. These calibration solutions are used to determine instrument response of the analytes relative to the internal quantitation standards and of the internal quantitation standards relative to the internal recovery standard.

Lipid - The organic solvent extractable constituents of adipose tissue consisting of fatty oils, proteins, and carbohydrates. The concentrations of PCDDs and PCDFs are reported on the lipid content bases.

Instrumental mass calibration - An internal instrumental systems check and tuning standard, perfluorokerosene (PFK), is introduced automatically by the instrument. The mass ion 380.976 is monitored by the analyst as an instrumental systems check and is also used to tune the instrument.

Internal quantitation standards - Carbon-13 labeled PCDDs and PCDFs, which are added to every sample and are present at the same concentration in every method blank and quality control sample. These are added to the adipose tissue prior to extraction and are used to measure the concentration of each analyte. The concentration of each internal quantitation standard is measured in every sample, and percent recovery is determined using the internal recovery standard.

Internal recovery standard - $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,3,7,8,9-HxCDD which is added to every sample extract just before the final concentration step and HRGC/MS-SIM analysis.

Limit of detection (LOD) - A value, derived from the noise to signal response, which is equal to 2.5 times the average instrumental noise level is the limit of detection.

Limit of quantitation (LOQ) - A value, derived from the noise to signal response, which is equal to 10 times the average instrumental noise level is the limit of quantitation.

Mass resolution check - Standard method used to demonstrate static resolution of 10,000 minimum (10% valley definition).

Not detected (ND) - A nonresponse or a response which is less than the limit of detection is reported as not detected.

Precision - The results from analysis of replicate samples (spiked and unspiked) provide the measure of method precision. The precision of the method is reported as standard deviation or relative standard deviation.

Performance check mixture, HRGC column - A mixture containing known amounts of selected TCDD standards; it is used to demonstrate continued acceptable performance of the capillary column, to separate (≤ 25 % valley on a 50-m CP Sil 88 or 60-m SP-2330 HRGC column and 30 to 60% for a 60 m DB-5 HRGC column) 2,3,7,8-TCDD isomer from all other 21 TCDD isomers, and to define the TCDD retention time window.

PCDD - Polychlorinated dibenzo-p-dioxins.

PCDF - Polychlorinated dibenzofurans.

Relative response factor - Response of the mass spectrometer to a known amount of an analyte relative to a known amount of an internal standard (quantitation or recovery).

Trace (TR) - A response which is greater than the limit of detection but less than the limit of quantitation is reported as a trace value. An estimated method detection limit is provided for trace value.

VIII. REFERENCES

1. Stanley JS. 1984. Methods of analysis of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in biological matrices--literature review and recommendations. EPA-560/584-00.

2. Stanley JS, Going JE, Redford DP, Kutz KW, Young AL. 1985. A survey of analytical methods for measurement of polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) in human adipose tissues. In: Chlorinated dioxins and dibenzofurans in the total environment II. Keith LH, Rappe C, Choudhary G, eds. Butterworth Publishers, pp. 181-195.

3. Stanley JS. 1984 (March 28). Proposed analytical method for analysis of PCDDs/PCDFs in human adipose tissue: special report. Washington, DC: Office of Pesticides and Toxic Substances, U.S. Environmental Protection Agency. Contract 68-02-3938, Work Assignment 8.

4. Stanley JS. 1986 (April 23). Broad scan analysis of human adipose tissue: polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Draft final report. Washington, DC: Office of Pesticides and Toxic Substances, U.S. Environmental Protection Agency. Contract 68-02-3938, Work Assignment 8.

5. Albro et al. 1985. Methods for the quantitative determination of multiple, specific polychlorinated dibenzo-p-dioxin and dibenzofuran isomers in human adipose tissue in the parts-per-trillion range. An interlaboratory study. Anal Chem 57: 2717-2725.

6. Patterson DG et al. 1986. High-resolution gas chromatographic/high-resolution mass spectrometric analysis of human adipose tissue for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Anal Chem 58: 705-713.

7. Schecter A, Tiernan TO, Taylor ML, VanNess GF, Barrett JH, Wagel DJ, Gitlitz G, Bogdasarian M. 1985. Biological markers after exposure to polychlorinated dibenzo-p-dioxins, dibenzofurans, biphenyls, and biphenylenes. Part I: Findings using fat biopsies to estimate exposure. In: Chlorinated dioxins and dibenzofurans in the total environment II. Keith LH, Rappe C, Choudhary G, eds. Butterworth Publishers, pp. 215-246.

8. Schecter A, Ryan JJ. 1985. Dioxin and furan levels in human adipose tissue from exposed and control populations. 189th National ACS Meeting Symposium on Chlorinated Dioxins and Dibenzofurans in the Total Environment III, Miami, Florida. Preprint Division of Environmental Chemistry, ACS 25:160-163, Paper No. 56.

9. Ryan JJ, Williams DT, Lau BPY, Sakuma T. 1985. Analysis of human fat tissue for 2,3,7,8-tetrachlorodibenzo-p-dioxin and chlorinated dibenzofuran residues. In: Chlorinated dioxins and dibenzofurans in the total environment II. Keith LH, Rappe C, Choudhary G, eds. Butterworth Publishers, pp. 205-214.

10. Ryan JJ, Schecter A, Lizotte R, Sun W-F, Miller L. 1985. Tissue distribution of dioxins and furans in humans from the general population. Chemosphere 14: 929-932.

11. Nygren M, Hansson M, Rappe C, Domellof L, Hardell L. 1985. Analysis of polychlorinated dibenzo-p-dioxins and dibenzofurans in adipose tissue from soft-tissue sarcoma patients and controls. 189th National ACS Meeting Symposium on Chlorinated Dioxins and Dibenzofurans in the Total Environment III, Miami, Florida, 1985. Preprint Division of Environmental Chemistry, ACS 25:160-163, Paper No. 55.

12. Smith LM, Stalling DL, Johnson JJ. 1984. Determination of part per trillion levels of polychlorinated dibenzofurans and dioxins in environmental samples. Anal Chem 58: 1830-1842.

13. Rappe C, Nygren M, Linström G, Hanson H. 1985. Dioxins and dibenzofurans in human tissues and milk of European origin. 5th International Symposium on Chlorinated Dioxins and Related Compounds, Bayreuth, FRG, September 16-19, 1985.

14. Ryan JJ. 1985. Variation of dioxins and furans in humans with age and organ by country. 5th International Symposium on Chlorinated Dioxins and Related Compounds, Bayreuth, FRG, September 16-19, 1985.

15. Graham M, Hileman FD, Wendling J, Wilson JD. 1985. Chlorocarbons in adipose tissue samples. 5th International Symposium on Chlorinated Dioxins and Related Compounds, Bayreuth, FRG, September 16-19, 1985.

16. Patterson DG, Holler JS, Smith SJ, Liddle JA, Sampson EJ, Needham LL. 1985. Human tissue data in certain U.S. populations. 5th International Symposium on Chlorinated Dioxins and Related Compounds, Bayreuth, FRG, September 16-19, 1985.

17. Patterson DG, Holler JS, Groce DF, Alexander LR, Lapeza CR, O'Conner RC, Liddle JA. 1986. Control of interferences in the analysis of human adipose tissue to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Environ Toxicol Chem 5: 355-360.

18. Holler JS, Patterson DG, Alexander LR, Groce DF, O'Connor RC, Lapeza CR. 1985. Control of artifacts and contamination in the development of a dioxin analytical program. 33rd Annual Conference on Mass Spectrometry and Allied Topics, San Diego, CA, May 26-31, 1985.

19. O'Keefe PN, Meyer C, Dillon K. 1982. Comparison of concentration techniques for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Anal Chem 54: 2623-2625.

APPENDIX A

ANALYTICAL PROTOCOL FOR DETERMINATION OF PCDDs AND PCDFs IN HUMAN ADIPOSE TISSUE

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ANALYTICAL PROTOCOL FOR DETERMINATION OF PCDDs AND PCDFs IN HUMAN ADIPOSE TISSUE

1. SCOPE AND APPLICATION

1.1 This method provides procedures for the detection and quantitative measurement of polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) at concentrations ranging from 1 to 100 pg/g for the tetrachloro congeners up to 5 to 500 pg/g for the octachloro congeners in 10-g aliquots of human adipose tissue.

1.2 The minimum measurable concentration is estimated to range from 1 pg/g (1 part per trillion) for 2,3,7,8-TCDD and 2,3,7,8-TCDF up to 5 pg/g for OCDD and OCDF. However, these detection limits depend on the kinds and concentrations of interfering compounds in the sample matrix and the absolute method recovery.

1.3 The method will be used to determine PCDDs and PCDFs, particularly congeners with chlorine substitution in the 2,3,7,8 positions. Table 1 lists the specific PCDDs and PCDFs and target method detection limits.

2. SUMMARY OF METHOD

Figure 1 presents a schematic of the analytical procedures for determination of PCDDs and PCDFs in human adipose tissue. The analytical method requires extraction and isolation of lipid materials from human adipose samples. This is accomplished using sample sizes ranging up to 10 g. The tissue is spiked with known amounts of the carbon-13 labeled PCDDs and PCDFs (e.g., 500 pg of $^{13}C_{12}$-TCDD/F to 2,500 pg of $^{13}C_{12}$-OCDD/F) as internal quantitation standards. Extraction and homogenization are accomplished using methylene chloride and a Tekmar Tissuemizer®. The extract is filtered through anhydrous sodium sulfate to remove water. The extraction procedure is repeated (three to five times) until the tissue sample has been thoroughly homogenized. The final extract is adjusted to a known volume (100 mL) and the extractable lipid is determined using a minimum of 1% of the final volume. The methylene chloride in the remaining extract is concentrated until only an oily residue remains. The residue is diluted with hexane (~ 200 mL), and 100 g of sulfuric acid modified silica gel (40% w/w) is added to the solution with stirring. The mixture is stirred for approximately 2 h, and the supernatant is decanted and filtered through anhydrous sodium sulfate. The adsorbent is washed with at least two additional aliquots of hexane.

The combined hexane extracts are eluted through a column consisting of a layer of sulfuric acid modified silica gel, and a layer of unmodified silica gel. The eluate is concentrated to approximately 1 mL and added to a column of acidic alumina. The PCDDs and PCDFs are eluted from the alumina using 20% methylene chloride/hexane. This eluate is concentrated to approximately 0.5 mL and is added to a 500-mg Carbopak C/Celite column. The PCDDs and PCDFs are eluted from the column using 20 mL of toluene.

Table 1. Target PCDD and PCDF Congeners and Target Method Detection Limits

Compound	CAS no.[a]	Target method detection limit (pg/g)[b]
2,3,7,8-TCDD	1746-01-6	1.0
2,3,7,8-TCDF	51207-31-9	1.0
1,2,3,7,8-PeCDD	40321-76-4	1.0
1,2,3,7,8-PeCDF	57117-41-6	1.0
2,3,4,7,8-PeCDF	57117-31-4	1.0
1,2,3,4,7,8-HxCDD	39227-28-6	2.5
1,2,3,6,7,8-HxCDD	57653-85-7	2.5
1,2,3,7,8,9-HxCDD	19408-74-3	2.5
1,2,3,4,7,8-HxCDF	70648-29-9	2.5
1,2,3,6,7,8-HxCDF	57117-44-9	2.5
1,2,3,7,8,9-HxCDF	72918-21-9	2.5
2,3,4,6,7,8-HxCDF	60851-34-5	2.5
1,2,3,4,6,7,8-HpCDD	35822-46-9	2.5
1,2,3,4,6,7,8-HpCDF	67562-39-4	2.5
1,2,3,4,7,8,9-HpCDF	55673-89-7	2.5
OCDD	3268-87-9	5.0
OCDF	39001-02-0	5.0

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[a] Chemical Abstract Services number.
[b] pg/g = parts per trillion.

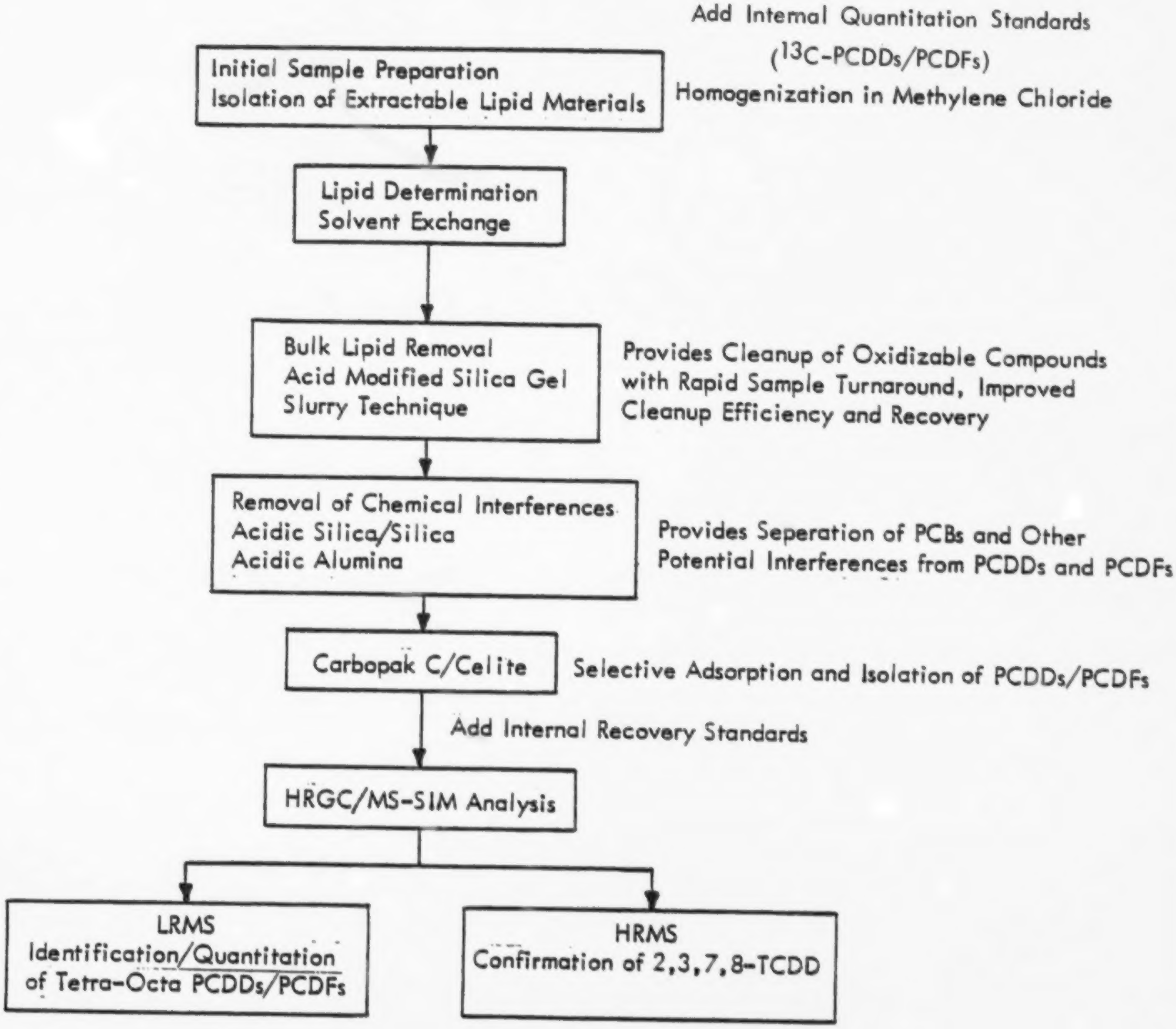

Add Internal Quantitation Standards
(^{13}C-PCDDs/PCDFs)
Homogenization in Methylene Chloride
Initial Sample Preparation
Isolation of Extractable Lipid Materials
Lipid Determination
Solvent Exchange
Bulk Lipid Removal
Acid Modified Silica Gel
Slurry Technique
Provides Cleanup of Oxidizable Compounds with Rapid Sample Turnaround, Improved Cleanup Efficiency and Recovery
Removal of Chemical Interferences
Acidic Silica/Silica
Acidic Alumina
Provides Seperation of PCBs and Other Potential Interferences from PCDDs and PCDFs
Carbopak C/Celite
Selective Adsorption and Isolation of PCDDs/PCDFs
Add Internal Recovery Standards
HRGC/MS-SIM Analysis
LRMS
Identification/Quantitation of Tetra-Octa PCDDs/PCDFs
HRMS
Confirmation of 2,3,7,8-TCDD

Figure 1. Schematic of the sample preparation and instrumental analysis procedures for determination of PCDDs and PCDFs in human adipose tissue.

The toluene is concentrated to less than 1 mL and transferred to conical vials. Tridecane (10 µL) containing 500 pg of an internal recovery standard is added as a keeper, and the extract is concentrated to final volume.

The HRGC/MS analysis is completed in the selected ion monitoring mode (SIM). Analysis of the tetra- through octachloro PCDD and PCDF congeners is achieved using low resolution mass spectrometry. Separation of the tetra- through octachloro PCDD and PCDF congeners is achieved using a 60-m DB-5 column. Verification of the 2,3,7,8-TCDD is achieved using either a 50-m CP Sil 88 column or 60-m SP-2330 column and HRGC/MS-SIM analysis in the high resolution mode (R = 10,000).

3. DEFINITIONS

3.1 Concentration calibration solutions -- Solutions containing known amounts of the native analytes (unlabeled 2,3,7,8-substituted PCDDs and PCDFs), the internal quantitation standards (Carbon-13 labeled PCDDs and PCDFs), and the recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD. These calibration solutions are used to determine instrument response of the analytes relative to the internal quantitation standards and of the internal quantitation standards relative to the internal recovery standard.

3.2 Internal quantitation standards -- Carbon-13 labeled PCDDs and PCDFs, which are added to every sample and are present at the same concentration in every method blank and quality control sample. These are added to the adipose tissue and are used to measure the concentration of each analyte. The concentration of each internal quantitation standard is measured in every sample, and percent recovery is determined using the internal recovery standard.

3.3 Internal recovery standard -- $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,3,7,8,9-HxCDD which is added to every sample extract just before the final concentration step and HRGC/MS-SIM analysis.

3.4 Laboratory method blank -- This blank is prepared in the laboratory through performing all analytical procedures except addition of a sample aliquot to the extraction vessel. A minimum of one laboratory method blank will be analyzed with each batch of samples.

3.5 HRGC column performance check mixture -- A mixture containing known amounts of selected TCDD standards; it is used to demonstrate continued acceptable performance of the capillary column, to separate ($\leq$ 25% valley on a 50-m CP Sil 88 or 60-m SP-2330 HRGC column and 30 to 60% for a 60-m DB-5 HRGC column) 2,3,7,8-TCDD isomer from all other 21 TCDD isomers, and to define the TCDD retention time window.

3.6 Relative response factor -- Response of the mass spectrometer to a known amount of an analyte relative to a known amount of an internal standard (quantitation or recovery).

3.7 Mass resolution check -- Standard method used to demonstrate static resolution of 10,000 minimum (10% valley definition).

3.8 Sample batch -- A sample batch consists of up to 10 human adipose tissue samples, one method blank, 2 internal quality control (QC) samples (spiked and unspiked), and an external performance audit sample (blind spike).

4. INTERFERENCES

Chemicals which elute from the HRGC column with ± 10 scans of the internal and/or recovery standards and which produce within the retention time window ions at any of the masses used to detect or quantify PCDDs, PCDFs, or the internal quantitation and recovery standards are potential interferences. Most frequently encountered potential interferences are other sample components that are extracted along with the PCDDs and PCDFs, e.g., PCBs, chlorinated methoxybiphenyls, chlorinated hydroxydiphenyl ethers, chlorinated benzylphenyl ethers, chlorinated naphthalenes, DDE, DDT, etc. The actual incidence of interference by these chemicals depends also upon relative concentrations, mass spectrometric resolution, and chromatographic conditions. Because very low levels (pg/g) of PCDDs and PCDFs are anticipated, the elimination of interferences is essential. High purity reagents and solvents must be used and all equipment must be scrupulously cleaned. Laboratory method blanks must be analyzed to demonstrate absence of contamination that would interfere with measurement of the PCDDs and PCDFs. Column chromatographic procedures are used to remove coextracted sample components; these procedures must be performed carefully to minimize loss of PCDDs and PCDFs during attempts to increase their concentration relative to other sample components.

5. SAFETY

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. The 2,3,7,8-TCDD is a known teratogen, mutagen, and carcinogen. Ingestion of microgram quantities can result in toxic effects. The other 2,3,7,8-substituted PCDDs and PCDFs may exhibit teratogenic, mutagenic, and carcinogenic effects. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. Only experienced personnel will be allowed to work with these chemicals.

5.2 All laboratory personnel will be required to wear laboratory coats or coveralls, gloves, and safety glasses. The neat standards, stock, and working solutions will be handled only in a Class A fume hood or glove box. When manipulating stock standards or working solutions, the analyst is advised to place the solution vials in a secure holder (sample block or glass beaker) to prevent accidental spills.

5.3 If these standards are spilled, absorb as much as possible with absorbent paper and place in a container clearly labeled as PCDD or PCDF waste. Solvent-wash all contaminated surfaces with toluene and absorbent paper followed by washing with a strong soap and water solution. Dispose of all contaminated materials in sealed steel containers labeled as contaminated with PCDD and/or PCDF residue and indicate the approximate level of contamination. As a final precaution, prepare a wipe sample of the exposed surface area and include the wipe as part of the sample analysis batch. This will be used to confirm that the work area is free of contamination.

5.4 If handling of these compounds results in skin contact, immediately remove all contaminated clothing and wash the affected skin areas with soap and water for at least 15 min.

5.5 Disposal of laboratory wastes -- All laboratory wastes (solvents and absorbents) will be disposed of as hazardous wastes. The laboratory personnel should take care to dispose of the sodium sulfate, silica gel, and alumina in separate containers. Excess solvents should be disposed of in gallon polyethylene jugs containing a layer of activated charcoal. Excess solvent that is known to be contaminated with PCDDs or PCDFs should be kept at a minimum by evaporating the solvent with a stream of air.

6. APPARATUS AND EQUIPMENT

6.1 High Resolution Gas Chromatograph/Mass Spectrometer/Data System (HRGC/HRMS/DS)

6.1.1 The GC must be equipped for temperature programming, and all required accessories must be available, such as syringes, gases, and a capillary column. The GC injection port must be designed for capillary columns. The use of splitless injection techniques is recommended. When using this method, a 1-µL injection volume is used. The injection volumes for all extracts, blanks, calibration solutions, and the performance check sample must be consistent.

6.1.2 High Resolution Gas Chromatograph-Mass Spectrometer Interface

The HRGC/MS interface is directly coupled to the mass spectrometer ion source. All components of the interface should be glass or glass-lined stainless steel. The interface components should be compatible with 300°C temperatures. The HRGC/MS interface must be appropriately designed so that the separation of the PCDDs and PCDFs which is achieved in the gas chromatographic column is not appreciably degraded. Cold spots and/or active surfaces (adsorption sites) in the HRGC/MS interface can cause peak tailing and peak broadening. It is recommended that the HRGC column be fitted directly into the MS ion source. Graphite ferrules should be avoided in the HRGC injection port since they may absorb PCDDs or PCDFs. Vespel or equivalent ferrules are recommended.

6.1.3 Mass Spectrometer

The mass spectrometer must be capable of maintaining a minimum resolution of 10,000 (10% valley) for high resolution confirmation analysis. The mass spectrometer must be operated in a selected ion monitoring (SIM) mode with total cycle time (including voltage reset time) of 1 s or less.

6.1.4 Data System

A dedicated hardware or data system is required to control the rapid multiple ion monitoring process and to acquire the data. Quantification data (peak areas or peak heights) and SIM traces (displays of intensities of each m/z (characteristic ion) being monitored as a function of time) must be acquired during the analyses. Quantifications may be reported based upon computer-generated peak areas or upon measured peak heights.

6.2 HRGC Columns

For isomer-specific determinations of 2,3,7,8-TCDD, the following fused silica capillary columns are recommended: a 50-m CP-Sil 88 column and a 60-m SP-2330 (SP-2331) column. However, any capillary column which separates 2,3,7,8-TCDD from all other TCDDs may be used for such analyses, provided that the minimum acceptance criteria in Section 8 are met.

6.3 Miscellaneous Equipment

6.3.1 Nitrogen evaporation apparatus with variable flow rate.

6.3.2 Balance capable of accurately weighing to ± 0.01 g.

6.3.3 Balance capable of accurately weighint to ± 0.0001 g.

6.3.4 Water bath -- equipped with concentric ring cover and capable of being temperature-controlled.

6.3.5 Stainless steel spatulas or spoons.

6.3.6 Magnetic stirrers and stir bars.

6.3.7 High speed tissue homogenizer -- Tekmar Tissuemizer® equipped with an EN-8 probe or equivalent.

6.3.8 Vacuum dessicator.

6.4 Glassware

6.4.1 Erlenmeyer flask -- 500 mL.

6.4.2 Kuderna-Danish apparatus -- 500-mL evaporating flask, 15-mL graduated concentrator tubes with ground-glass stoppers, and three-ball macro Snyder column (Kontes K-570001-0500, K-503000-0121, and K-569001-0219 or equivalent).

6.4.3 Minivials -- 1-mL borosilicate glass with conical-shaped reservoir and screw caps lined with Teflon®-faced silicone disks.

6.4.4 Powder funnels -- glass.

6.4.5 Chromatographic columns for the silica and alumina chromatography -- 1 cm ID x 10 cm long and 1 cm ID x 30 cm long with 250-mL reservoir and equipped with TFE stopcocks.

6.4.6 Chromatographic column for the Carbopak cleanup -- disposable 5-mL graduated glass pipets, 6 to 7 mm ID.

6.4.7 Glass rods.

6.4.8 Carborundum boiling chips -- Extracted for 6 hr in a Soxhlet apparatus with benzene and air dried.

6.4.9 Glass wool, silanized (Supelco) -- Extract with methylene chloride and hexane and air dry before use.

6.4.10 Glassware cleaning procedure -- All glassware used for these analyses will be cleaned via the following procedure. Wash the glassware in soap and water, rinse with copious amounts of tap water, distilled water, and distilled-in-glass acetone, in that order. Immediately prior to use, the glassware should be rinsed with distilled-in-glass quality solvents: methylene chloride, toluene, and hexane. The glassware should be allowed to dry fully.

As an added precuation, all glassware will be marked with a unique code that should be noted in the extraction and cleanup procedures for each sample. This glassware tracking will allow background results from specific glassware to be documented.

After use, each piece of glassware should be rinsed with the last solvent used in it, followed by a rinse with toluene, then acetone, before transferring it to the glassware washing facility.

7. REAGENTS AND STANDARD SOLUTIONS

7.1 Column Chromatography Reagents

7.1.1 Alumina, acidic (Biorad, AG-4) -- Extract the alumina in a Soxhlet apparatus with methylene chloride for 18 h (minimum of two cycles per hour). Air dry and activate it by heating in a foil-covered glass container for 24 h at 190°C.

7.1.2 Silica gel -- High purity grade, type 60, 70-230 mesh; extract the silica gel in a Soxhlet apparatus with methylene chloride for 10 h (minimum of 2 cycles per hour). Air dry and activate it by heating in a foil-covered glass container for 24 h at 130°C.

7.1.3 Silica gel impregnated with 40% (by weight) sulfuric acid -- Add two parts (by weight) concentrated sulfuric acid to three parts (by weight) silica gel (extracted and activated) (e.g., 40 g of H_2SO_4 plus 60 g of silica gel) in a glass screw-cap bottle. Tumble for 5 to 6 h, shaking occasionally until free of lumps.

7.1.4 Sulfuric acid, concentrated -- ACS grade, specific gravity 1.84.

7.1.5 Graphitized carbon black (Carbopack C, Supelco), surface of approximately 12 m^2/g, 80/100 mesh -- Mix thoroughly 3.6 g of Carbopack C and 16.4 g of Celite 545® in a 40-mL vial. Activate at 130°C for 6 h. Store in a desiccator.

7.1.6 Celite 545® (Fischer Scientific), reagent grade, or equivalent.

7.2 Desiccating agents -- Sodium sulfate; granular, anhydrous. Before use extract with methylene chloride for 16 h (minimum of two cycles per hour), air dry and then muffle for ≥ 4 h in a shallow tray at 400°C. Let it cool in a desiccator and store in oven at 130°C.

7.3 Solvents -- High purity, distilled in glass: methylene chloride, toluene, benzene, cyclohexane, methanol, acetone, hexane; reagent grade: tridecane. High purity solvents are dispensed from Teflon® squirt bottles.

7.4 Concentration Calibration Solutions (Table 2)

Eight tridecane solutions containing native calibration standards, $^{13}C_{12}$-labeled internal quantitation standards, and two internal recovery standards are required. The complete compound list is

Table 2. Concentration Calibration Solutions

Compound	Concentration in calibration solutions in pg/μL							
Native	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8
2,3,7,8-TCDD	200	100	50	25	10	5	2.5	1
2,3,7,8-TCDF	200	100	50	25	10	5	2.5	1
1,2,3,7,8-PeCDD	200	100	50	25	10	5	2.5	1
1,2,3,7,8-PeCDF	200	100	50	25	10	5	2.5	1
2,3,4,7,8-PeCDF	200	100	50	25	10	5	2.5	1
1,2,3,4,7,8-HxCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,6,7,8-HxCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,7,8,9-HxCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,7,8-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,6,7,8-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,7,8,9-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
2,3,4,6,7,8-HxCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,6,7,8-HpCDD	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,6,7,8-HpCDF	500	250	125	62.5	25	12.5	6.25	2.5
1,2,3,4,7,8,9-HpCDF	500	250	125	62.5	25	12.5	6.25	2.5
OCDD	1,000	500	250	125	50	25	12.5	5
OCDF	1,000	500	250	125	50	25	12.5	5
Internal Quantitation Standards								
$^{13}C_{12}$-2,3,7,8-TCDD	50	50	50	50	50	50	50	50
$^{13}C_{12}$-2,3,7,8-TCDF	50	50	50	50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8-PeCDD	50	50	50	50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8-PeCDF	50	50	50	50	50	50	50	50
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	125	125	125	125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	125	125	125	125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	125	125	125	125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF	125	125	125	125	125	125	125	125
$^{13}C_{12}$-OCDD	250	250	250	250	250	250	250	250
Internal Recovery Standard								
$^{13}C_{12}$-1,2,3,4-TCDD	50	50	50	50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8,9-HxCDD	125	125	125	125	125	125	125	125

given in Table 2. The native 2,3,7,8-TCDD is supplied as a certified standard solution from the U.S. EPA QA Reference Materials Branch. All other native compounds were supplied in crystalline form by Cambridge Isotope Laboratories (Woburn, MA). $^{13}C_{12}$-Labeled internal quantitation standards were supplied in solution in n-nonane by Cambridge Isotope Laboratories. Portions of the native standards were accurately weighed to the nearest 0.001 mg with a Cahn 27 electrobalance and dissolved in toluene.

7.5 Column Performance Check Mixture

The column performance check mixture consists of several TCDD isomers which will be used to document the separation of 2,3,7,8-TCDD from all other isomers. This solution will contain TCDDs (A) eluting closely to 2,3,7,8-TCDD, and the first- (F) and last-eluting (L) TCDDs.

Analyte	Approximate amount per ampule
Unlabeled 2,3,7,8-TCDD	10 ng
$^{13}C_{12}$-2,3,7,8-TCDD	10 ng
1,2,3,4-TCDD (A)	10 ng
1,4,7,8-TCDD (A)	10 ng
1,2,3,7-TCDD (A)	10 ng
1,2,3,8-TCDD (A)	10 ng
1,3,6,8-TCDD (F)	10 ng
1,2,8,9-TCDD (L)	10 ng

7.6 Spiking Solutions

Three solutions are prepared using the same stock as in Section 7.4. A native standard solution and a $^{13}C_{12}$ internal quantitation standard solution are prepared in isooctane (Tables 3 and 4). A recovery standard solution is prepared in tridecane (Table 4). Samples are spiked with 100 µL of internal quantitation standard solution and final sample extracts are spiked with 10 µL of internal recovery standard solution.

8. HIGH RESOLUTION GAS CHROMATOGRAPHY/MASS SPECTROMETRY PERFORMANCE CRITERIA

Samples and standards are analyzed by using a Carlo Erba MFC500 gas chromatography (GC) coupled to a Kratos MS50TC double-focusing mass spectrometer (MS) to be operated in the electron impact mode. The HRGC/MS interface is simply a direct connection of the fused silica HRGC column to the ion source of the MS via a heated interface oven. Data acquisition and processing are controlled by a Finnigan-MAT Incos 2300 data system.

Table 3. Native Spiking Solution[a]

Compound	Concentration (pg/μL)
2,3,7,8-TCDD	5
2,3,7,8-TCDF	5
1,2,3,7,8-PeCDD	5
1,2,3,7,8-PeCDF	5
2,3,4,7,8-PeCDF	5
1,2,3,4,7,8-HxCDD	12.5
1,2,3,6,7,8-HxCDD	12.5
1,2,3,7,8,9-HxCDD	12.5
1,2,3,4,7,8-HxCDF	12.5
1,2,3,6,7,8-HxCDF	12.5
1,2,3,7,8,9-HxCDF	12.5
2,3,4,6,7,8-HxCDF	12.5
1,2,3,4,6,7,8-HpCDD	12.5
1,2,3,4,6,7,8-HpCDF	12.5
1,2,3,4,7,8,9-HpCDF	12.5
OCDD	25
OCDF	25

[a] Prepared in isooctane.

Table 4. Internal Standard Spiking Solutions

Compound	Concentration (pg/μL)
Internal Quantitation Standards[a]	
$^{13}C_{12}$-2,3,7,8-TCDD	5
$^{13}C_{12}$-2,3,7,8-TCDF	5
$^{13}C_{12}$-1,2,3,7,8-PeCDD	5
$^{13}C_{12}$-1,2,3,7,8-PeCDF	5
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	12.5
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	12.5
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	12.5
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF	12.5
$^{13}C_{12}$-OCDD	25
Internal Recovery Standard[b]	
$^{13}C_{12}$-1,2,3,4-TCDD	50
$^{13}C_{12}$-1,2,3,7,8,9-HxCDD	125

[a] Prepared in isooctane.
[b] Prepared in tridecane.

8.1 HRGC/MS Analysis of PCDD/PCDF

Single run selected ion monitoring (SIM) analysis of the tetrachloro through octachloro-dioxins and furans is carried out with the instrumental conditions and parameters outlined in Table 5. For each HRGC/MS run, five distinct groups of ions, which correspond to each chlorine level, are sequentially monitored. These ion descriptors are shown in Table 6. The masses of the two most abundant ions in the molecular ion cluster of each dioxin and furan and isotopically labeled standard are monitored. In addition, the masses corresponding to the molecular ions of the hexachloro through decachlorodiphenyl ethers (PCDEs) are monitored to aid in the confirmation of positive furan results. Interference from the presence of PCDE is noted by coincident response to the characteristic ions for PCDFs. A lock mass, m/z 381 from PFK (perfluorokerosene), is used to observe and correct any magnet/instrument drift during the analysis.

8.1.1 Tuning and Mass Calibration

The mass spectrometer is tuned on a daily basis to yield optimum sensitivity and peak shape using an ion peak (m/z 381) from PFK. The resolution is visually monitored and maintained at ≥ 3,000 (10% valley definition) to provide adequate noise rejection while maintaining good ion transmission.

Mass calibration of the mass spectrometer for the HRGC/MS analysis of PCDD/PCDF is carried out on a daily basis. The magnetic field is adjusted to pass m/z 300 at full accelerating voltage. PFK is admitted to the MS and an accelerating voltage scan from 8,000 to 4,000 V is acquired by the data system. This corresponds to an effective mass range of 301 to 593 amu. Upon completion of a successful calibration step, the five ion descriptors shown in Table 6 are updated to reflect the new mass calibration.

8.1.2 Ion Descriptor Switching

The ion descriptors shown in Table 6 are sequentially monitored during a PCDD/PCDF analysis to cover the retention windows of each chlorination level. The retention windows and hence the descriptor switch points are determined initially and whenever a new HRGC column is installed by injection of a mixture of PCDD and PCDF congeners. Daily adjustment of the descriptor switch times are performed when careful monitoring of the standard retention times shows this to be necessary. The descriptors are designed to ensure acquisition of all isomers of each homolog.

Table 5. HRGC/LRMS Operating Conditions for PCDD/PCDF Analysis

Mass spectrometer

Accelerating voltage:	8,000 V
Trap current:	500 μA
Electron energy:	70 eV
Electron multiplier voltage:	-1,800 V
Source temperature:	280°C
Resolution:	≧ 3,000 (10% valley definition)
Overall SIM cycle time:	1 s

Gas chromatograph

Column coating:	DB-5
Film thickness:	0.25 μm
Column dimensions:	60 m x 0.25 mm ID
He linear velocity:	~ 25 cm/sec
He head pressure:	1.75 kg/cm^2 (25 psi)
Injection type:	Splitless, 45 s
Split flow:	30 mL/min
Purge flow:	6 mL/min
Injector temperature:	270°C
Interface temperature:	300°C
Injection size:	1-2 μL
Initial temperature:	200°C
Initial time:	2 min
Temperature program:	200°C to 330°C at 5°C/min

Table 6. Ions Monitored for HRGC/MS of PCDD/PCDF

Descriptor	ID	Mass	Nominal dwell time (sec)
A1	TCDF	303.902	0.090
		305.899	0.090
	$^{13}C_{12}$-TCDF	315.942	0.090
		317.939	0.090
	TCDD	319.897	0.090
		321.894	0.090
	$^{13}C_{12}$-TCDD	331.937	0.090
		333.934	0.090
	HxCDPE	373.840	0.090
	PFK (lock mass)	380.976	0.090
A2	TCDF	303.902	0.045
		305.899	0.045
	TCDD	319.897	0.045
		321.894	0.045
	PeCDF	337.863	0.045
		339.860	0.045
	$^{13}C_{12}$-PeCDF	349.903	0.045
		351.900	0.045
	PeCDD	353.858	0.045
		355.855	0.045
	$^{13}C_{12}$-PeCDD	365.898	0.045
		367.895	0.045
	PFK (lock mass)	380.976	0.035
	HpCDPE	407.801	0.035
A3	HxCDF	373.821	0.080
		375.818	0.080
	PFK (lock mass)	380.976	0.080
	$^{13}C_{12}$-HxCDF	385.861	0.080
		387.858	0.080
	HxCDD	389.816	0.080
		391.813	0.080
	$^{13}C_{12}$-HxCDD	401.856	0.080
		403.853	0.080
	OCDPE	443.759	0.080

Table 6 (continued)

Descriptor	ID	Mass	Nominal dwell time (sec)
A4	PFK (lock mass)	380.976	0.040
	HxCDD	389.816	0.040
		391.813	0.040
	HpCDF	407.782	0.040
		409.779	0.040
	$^{13}C_{12}$-HpCDF	419.822	0.040
		421.819	0.040
	HpCDD	423.777	0.040
		425.774	0.040
	$^{13}C_{12}$-HpCDD	435.817	0.040
		437.814	0.040
	$^{37}Cl_4$-HpCDD	429.768	0.040
		431.765	0.040
	NCDPE	477.720	0.040
A5	PFK (lock mass)	380.976	0.06
	OCDF	441.743	0.07
		443.740	0.07
	$^{13}C_{12}$-OCDF	453.783	0.07
		455.780	0.07
	OCDD	457.738	0.07
		459.735	0.07
	$^{13}C_{12}$-OCDD	469.779	0.07
		471.776	0.07
	DCDPE	511.681	0.06

8.1.3 HRGC Column Performance (60-m DB-5)

The HRGC column performance must be demonstrated at the start of each 12-h analysis period.

8.1.3.1 Inject 1 μL of the column performance check solution (Section 7.5) and acquire selected ion monitoring (SIM) data for m/z 320, 322, 332, and 334.

8.1.3.2 The chromatographic peak separation between 2,3,7,8-TCDD and the peaks representing any other TCDD isomers should be resolved with a valley of 30-60%, where

$$\text{Valley \%} = (x/y)(100)$$

x = measured height of the valley between the chromatographic peak corresponding to 2,3,7,8-TCDD and the peak of the nearest TCDD isomer; and

y = the peak height of 2,3,7,8-TCDD.

Figure 2 is an example of the separation of a TCDD isomer mixture and the calculation of isomer resolution.

It is the responsibility of the laboratory to verify the conditions suitable for the appropriate resolution of 2,3,7,8-TCDD from all other TCDD isomers. The column performance check solution also contains the TCDD isomers eluting first and last under the analytical conditions specified in this protocol, thus defining the retention time window for total TCDD determination. Any individual selected ion current profile or the reconstructed total ion current (m/z 320 + m/z 322) consititutes an acceptable form of data presentation.

8.1.4 Initial Calibration for PCDD/PCDF Analysis

Initial calibration is required before any samples are analyzed for PCDD/PCDF. Initial calibration is also required if any routine calibration does not meet the required criteria listed in Section 8.1.7.

8.1.4.1 Tune and calibrate the instrument with PFK as outlined in Section 8.1.1.

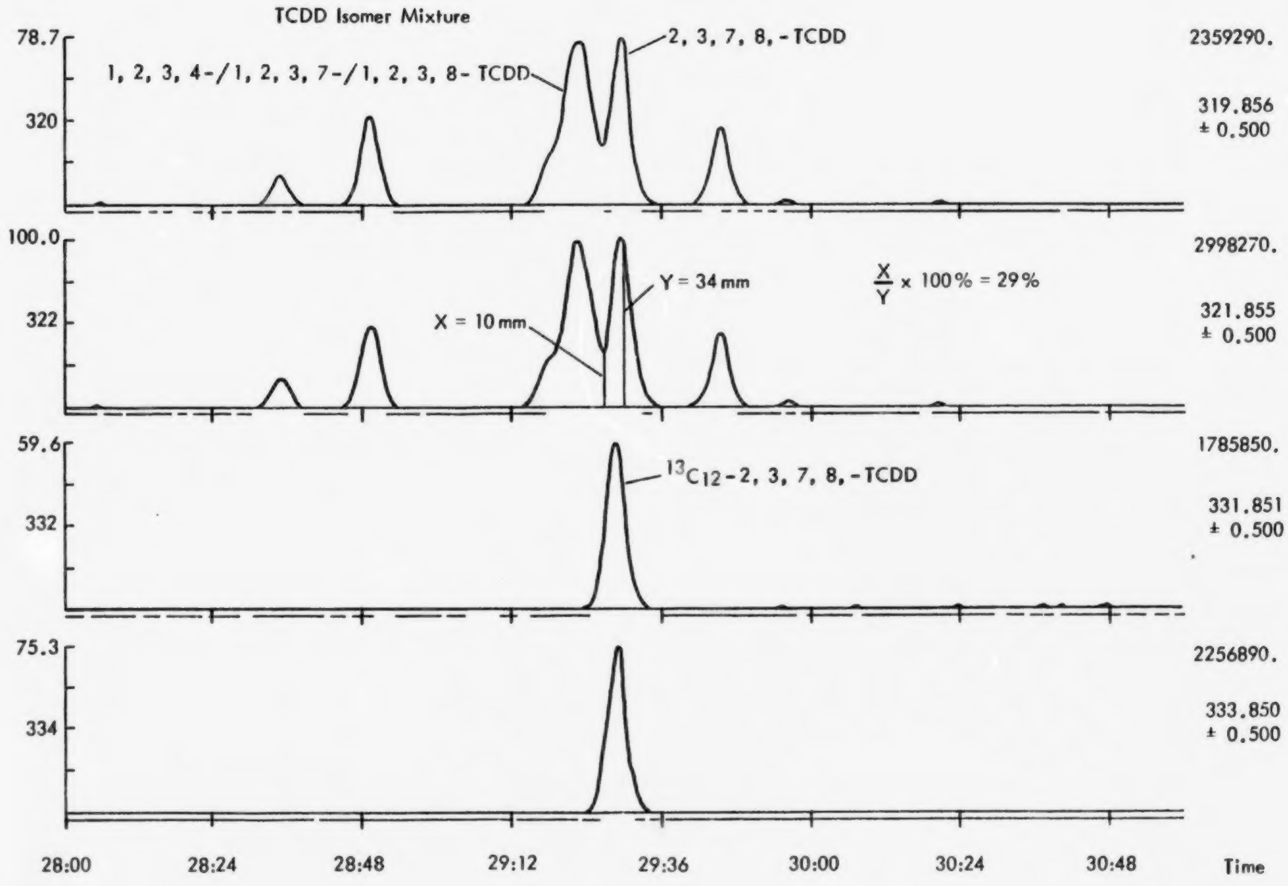


Figure 2. Example of the separation of 2,3,7,8-TCDD from other TCDD isomers on a 60 m DB-5 column.

8.1.4.2 Six of the eight concentration calibration solutions listed in Table 2 will be analyzed for the initial calibration phase. These must include solutions CS4 through CS8 (Table 2). The analyst may select any of the remaining solutions for demonstrating calibration at the upper concentration range.

8.1.4.3 Using the HRGC and MS conditions in Table 5 and the SIM monitoring descriptors in Table 6, analyze a 1-μL aliquot of each of the six concentration calibration solutions in triplicate.

8.1.4.4 Compute the relative response factors (RRFs) for each analyte in the concentration calibration solution using the criteria for positive identification of PCDD/PCDF's given in Section 14.1 and the computational methods in Section 14.2.

8.1.4.5 Compute the means and their respective relative standard deviations (% RSD) for the RRFs from each triplicate analysis for each analyte in the standard.

8.1.4.6 Calculate the grand means ($\overline{RRF}$) and their respective RSDs using the six mean RRFs for each analyte.

8.1.5 Criteria for Acceptable Initial Calibration

8.1.5.1 The % RSD for the response factors for each triplicate analysis of a single concentration calibration standard for each analyte must be less than ± 30% except for the TCDD and TCDF, which must be less than ± 20%.

8.1.5.2 The variation of the mean RRFs for the six concentration calibrated standards (Section 8.1.5.1) must be less than 30% except for the TCDD and TCDF which must be less than 20%.

8.1.5.3 The SIM traces for all ions used for quantitation must present a signal-to-noise (S/N) ratio of ≧ 2.5. This includes analytes and isotopically labeled standards.

8.1.5.4 Isotopic ratios must be within ± 20% of the theoretical values (see Table 7).

NOTE: If the criteria for acceptable calibration listed above have been met, the RRF can be considered independent of the analyte quantity for the calibration concentration range. The grand mean RRF from the initial calibration for unlabeled PCDD/PCDFs and for the isotopically labeled standards will be used for all calculations until routine calibration criteria (Section 8.1.7) are no longer met. At such time, new mean RRFs will be calculated from a new set of six triplicate determinations.

8.1.6 Routine Calibrations

Routine calibrations must be performed at the beginning of every day before actual sample analyses are performed and as the last injection of every day.

8.1.6.1 Inject 1 μL of the concentration calibration solution CS 7 (see Table 2) as the initial calibration check on each analysis day. It is recommended that the analyst select a concentration calibration solution that brackets the sample concentrations observed on a single analysis date as the last injection of each analysis date.

8.1.6.2 Compute the RRFs for each analyte in the concentration calibration solution using the criteria for positive identification of PCDD/Fs given in Section 14.1 and the computational methods in Section 14.2.

8.1.7 Criteria for Acceptable Routine Calibration

8.1.7.1 The measured RRF for all analytes must be within ± 30% of the grand mean values established by triplicate analysis of the calibration concentration solutions, except for TCDD and TCDF, which must be within ± 20% of the mean values established in the initial calibration step.

8.1.7.2 Isotopic ratios must be within ± 20% of the theoretical value for each analyte and isotopically labeled standard (see Table 7).

Table 7. Ion Ratios for HRGC/LRMS Analysis of PCDD/PCDF

Compound	Ions monitored	Theoretical ratio	Acceptable range
TCDF	304/306	0.76	0.61 - 0.91
$^{13}C_{12}$-TCDF	316/318	0.76	0.61 - 0.91
TCDD	320/322	0.76	0.61 - 0.91
$^{13}C_{12}$-TCDD	332/334	0.76	0.61 - 0.91
PeCDF	338/340	0.61	0.49 - 0.73
$^{13}C_{12}$-PeCDF	350/352	0.61	0.49 - 0.73
PeCDD	354/356	0.61	0.49 - 0.73
$^{13}C_{12}$-PeCDD	366/368	0.61	0.49 - 0.73
HxCDF	374/376	1.22	0.98 - 1.46
$^{13}C_{12}$-HxCDF	386/388	1.22	0.98 - 1.46
HxCDD	390/392	1.22	0.98 - 1.46
$^{13}C_{12}$-HxCDD	402/404	1.22	0.98 - 1.46
HpCDF	408/410	1.02	0.82 - 1.22
$^{13}C_{12}$-HpCDF	420/422	1.02	0.82 - 1.22
HpCDD	424/426	1.02	0.82 - 1.22
$^{13}C_{12}$-HpCDD	436/438	1.02	0.82 - 1.22
OCDF	442/444	0.87	0.70 - 1.04
$^{13}C_{12}$-OCDF	454/456	0.87	0.70 - 1.04
OCDD	458/460	0.87	0.70 - 1.04
$^{13}C_{12}$-OCDD	470/472	0.87	0.70 - 1.04

8.1.7.3 If any of the above criteria is not met, a second attempt may be made before repeating the entire initialization process.

8.2 HRGC/HRMS Analysis (Isomer Specific TCDD Analysis)

Isomer specific analysis for 2,3,7,8-TCDD is carried out with the instrumental conditions and parameters shown in Table 8. In addition to monitoring the masses of the most abundant molecular ions of TCDD, an ion corresponding to the loss of COCl from the molecular ion is monitored for verification purposes. Mass spectrometer resolution is maintained at or above 10,000 (10% valley definition) in order to increase the specificity of the analysis.

8.2.1 Tuning and Mass Calibration

8.2.1.1 The mass spectrometer must be operated in the electron (impact) ionization mode. Static resolving power of at least 10,000 (10% valley) must be demonstrated before any analysis of a set of samples is performed. Static resolution checks must be performed at the beginning and at the end of each 12-h period of operation. However, it is recommended that a visual check (i.e., not documented) of the static resolution be made before and after each analysis.

8.2.1.2 The MS shall be tuned daily using PFK to yield a resolution of at least 10,000 (10% valley) and optimal response at m/z 254.986. This step is followed by calibration of an accelerating voltage scan of PFK beginning at m/z 254 (typical calibration range is 255 to 493 amu). Other voltage scans from the same data file are used to establish and document both the resolution at m/z 316.983 and the mass measurement accuracy at m/z 330.979.

8.2.1.3 Following calibration, the SIM experiment descriptor is updated to reflect the new calibration. Six masses (see Table 8) are monitored by scanning ∼ m/10,000 amu (atomic mass units) over each mass. The total cycle time is kept to 1 s. The m/z 280.983 ion from PFK is used as a lock mass because it is the most abundant PFK ion within the range of m/z 255 to 334 and therefore permits the use of low partial pressures of PFK, which minimizes PFK interferences at the analytical masses.

Table 8. HRGC/HRMS Operating Conditions

Mass spectrometer

Accelerating voltage:	8,000 V
Trap current:	500 μA
Electron energy:	70 eV
Electron multiplier voltage:	2,000 V
Source temperature:	280°C
Resolution:	10,000 (10% valley definition)

SIM Parameters

Identity	Mass	Nominal dwell times (s)
TCDD-COCl	258.930	0.15
TCDD	319.897	0.15
TCDD	321.894	0.15
$^{13}C_{12}$-TCDD	331.937	0.15
$^{13}C_{12}$-TCDD	333.934	0.15
PFK (lock mass)	280.983	0.10

Overall SIM cycle time = 1 s

Gas chromatograph

Column coating:	CP-Sil 88
Film Thickness:	0.2 μm
Column dimensions:	50 m x 0.22 mm ID
Helium linear velocity:	∿ 25 cm/s
Helium head pressure:	1.75 kg/cm^2 (25 psi)
Injection type:	Splitless, 45 s
Split flow:	30 mL/min
Purge flow:	6 mL/min
Injector temperature:	270°C
Interface temperature:	240°C
Injection size:	2 μL
Initial temperature:	200°C
Initial time:	1 min
Temperature program:	200°C to 240°C at 4°C/min

8.2.2 Mass Measurement and Resolution Check

Using a PFK molecular leak, tune the instrument to meet the minimum required resolving power of 10,000 (10% valley) at m/z 254.986 (or any other mass reasonably close to m/z 259). Calibrate the voltage sweep at least across the mass range m/z 259 to m/z 334 and verify that m/z 330.979 from PFK (or any other mass close to m/z 334) is measured within ± 5 ppm (i.e., 1.7 mmu, if m/z 331 is chosen) using m/z 254.986 as a reference. Documentation of the mass resolution must then be accomplished by recording the peak profile of the PFK reference peak m/z 318.979 (or any other reference peak at a mass close to m/z 320/322). The format of the peak profile representation must allow manual determination of the resolution; i.e., the horizontal axis must be a calibrated mass scale (amu or ppm per division). The results of the peak width measurement (performed at 5% of the maximum which corresponds to the 10% valley definition) must appear on the hard copy and cannot exceed 100 ppm (or 31.9 mmu if m/z 319 is the chosen reference ion).

8.2.3 HRGC Column Performance (50-m CP Sil 88/60-m SP-2330)

Prior to any HRGC/HRMS analysis of calibration solutions or samples for 2,3,7,8-TCDD, the resolution of the HRGC columns must be documented to be within allowable limits in order to provide conditions adequate for unambiguous isomer-specific analysis of 2,3,7,8-TCDD. This column performance check must be demonstrated at the start of each 12-h analysis period.

8.2.3.1 Inject 2 µL of the column performance check solution and acquire selected ion monitoring (SIM) data for m/z 258.930, 319.897, 321.894, 331.937, and 333.934 within a total cycle time of $\leq$ 1 s (Table 8).

8.2.3.2 The chromatographic peak separation between 2,3,7,8-TCDD and the peaks representing any other TCDD isomers must be resolved with a valley of $\leq$ 25%, where

$$\text{Valley } \% = (x/y)(100)$$

x = measured height of the valley between the chromatographic peak corresponding to 2,3,7,8-TCDD and the peak of the nearest TCDD isomer; and

y = the peak height of 2,3,7,8-TCDD.

8.2.3.3 If the above resolution requirement is not met, corrective action must be taken and acceptable resolution documented prior to any further analyses. Corrective action may include removal of the first meter of the HRGC column, replacement or clearing of the injector port, or complete replacement of the GC column.

8.2.3.4 The column performance check solution also contains the TCDD isomers eluting first and last under the analytical conditions specified in this protocol, thus defining the retention time window for total TCDD determination. The peaks representing 2,3,7,8-TCDD and the first and the last eluting TCDD isomer should be labeled and identified as such on the chromatograms (F and L, respectively). Any individual selected ion current profile or the reconstructed total ion current (m/z 259 + m/z 320 + m/z 322) constitutes an acceptable form of data presentation.

8.2.4 Initial Calibration for HRGC/HRMS 2,3,7,8-TCDD Analysis

Initial calibration is required before any samples are analyzed for 2,3,7,8-TCDD. Initial calibration is also required if any routine calibration does not meet the required criteria listed in Section 8.2.6.

8.2.4.1 At least six of the concentration calibration solutions listed in Table 2 must be utilized for the initial calibration. These must include solutions CS4 through CS8. The analyst may select any of the remaining solutions for demonstrating calibration at the upper concentration range.

8.2.4.2 Tune and calibrate the instrument with PFK as described in Section 8.2.1.

8.2.4.3 Inject 1 μL of the column performance check solution (Section 8.2.3) and acquire SIM mass spectra data for m/z 258.930, 319.897, 321.894, 331.937, and 333.934 using a total cycle time of $\leq$ 1 s (see Table 8). The laboratory must not perform any further analysis until it has been demonstrated and documented that the criterion listed in Section 8.2.3.2 has been met.

8.2.4.4 Using the same GC and MS conditions (Table 8) that produced acceptable results with the column performance check solution, analyze a 1-µL aliquot of each of the six concentration calibration solutions in triplicate.

8.2.4.5 Calculate the RRFs for unlabeled 2,3,7,8-TCDD relative to $^{13}C_{12}$-2,3,7,8-TCDD and the RRF for $^{13}C_{12}$-2,3,7,8-TCDD relative to $^{13}C_{12}$-1,2,3,4-TCDD using the criteria for positive identification of TCDD by HRGC/HRMS given in Section 14.1 and the computational methods in Section 14.2.

8.2.4.6 Calculate the six means (RRFs) and their respective relative standard deviations (% RSD) for the response factors from each of the triplicate analyses for both unlabeled and $^{13}C_{12}$-2,3,7,8-TCDD.

8.2.4.7 Calculate the grand mean RRFs and their respective relative standard deviations (% RSD) using the six mean RRFs.

8.2.5 Criteria for Acceptable Initial Calibration

The criteria listed below for acceptable calibration must be met before analysis of any sample is performed.

8.2.5.1 The percent relative standard deviation (RSD) for the response factors from each of the triplicate analyses of a single concentration calibration standard for both unlabeled and $^{13}C_{12}$-2,3,7,8-TCDD must be less than 20%.

8.2.5.2 The variation of the mean RRFs from the six concentration calibration standards unlabeled and $^{13}C_{12}$-2,3,7,8-TCDD must be less than 20% RSD.

8.2.5.3 SIM traces for 2,3,7,8-TCDD must present a signal-to-noise ratio of ≧ 2.5 for m/z 258.930, m/z 319.897, and m/z 321.894.

8.2.5.4 SIM traces for $^{13}C_{12}$-2,3,7,8-TCDD must present a signal-to-noise ratio ≧ 2.5 for m/z 331.937 and m/z 333.934.

8.2.5.5 Isotopic ratios for 320/322 and 332/334 must be within the allowed range (0.61 to 0.91).

NOTE: If the criteria for acceptable calibration listed above have been met, the RRF can be considered independent of the analyte quantity for the calibration concentration range. The grand mean RRF from the initial calibration for unlabeled 2,3,7,8-TCDD and for $^{13}C_{12}$-2,3,7,8-TCDD will be used for all calculations until routine calibration criteria (Section 8.2.6) are no longer met. At such time, new mean RRFs will be calculated from a new set of six triplicate determinations.

8.2.6 Routine Calibrations

Routine calibrations must be performed at the beginning of a 12-h period after successful mass resolution and HRGC column performance check runs and before analysis of actual samples. The response factor calibration must also be verified at the end of each analysis date.

8.2.6.1 Inject 1 µL of the concentration calibration solution (CS7, Table 2) which contains 2.5 pg/µL of unlabeled 2,3,7,8-TCDD, 50.0 pg/µL of $^{13}C_{12}$-2,3,7,8-TCDD, and 50 pg/µL of $^{13}C_{12}$-1,2,3,4-TCDD. Using the same HRGC/MS/DS conditions as used in Table 8, determine and document acceptable calibration as provided below.

8.2.7 Criteria for Acceptable Routine Calibration

The following criteria must be met before further analysis is performed. If these criteria are not met, corrective action must be taken and the instrument must be recalibrated.

8.2.7.1 The measured RRF for unlabeled 2,3,7,8-TCDD must be within 20% of the mean values established in the initial calibration by triplicate analyses of concentration calibration solutions.

8.2.7.2 The measured RRF for $^{13}C_{12}$-2,3,7,8-TCDD must be within 20% of the mean value established by triplicate analysis of the concentration calibration solutions during the initial calibration.

8.2.7.3 Isotopic ratios must be within the allowed range (0.61 to 0.90).

8.2.7.4 If one of the above criteria is not satisfied, a second attempt can be made before repeating the entire initialization process.

NOTE: An initial calibration must be carried out whenever the routine calibration solution is replaced by a new one from a different lot.

9. QUALITY CONTROL PROCEDURES

9.1 Summary of QC Analyses

9.1.1 Initial and routine calibration and instrument performance checks.

9.1.2 Analysis of a batch of samples with accompanying QC analyses:

Sample batch -- 10 NHATS adipose tissue samples plus additional QC analyses including 1 method blank, a control tissue and a spiked tissue sample.

"Blind" QC (external QC) samples may be submitted by an external source (quality assurance group or independent laboratory) and included among the batch of samples. Blind samples include spiked samples, unidentified duplicates, and performance evaluation samples.

9.2 Performance Evaluation Solutions -- Included among the samples in every third batch will be a solution provided by the quality control coordinator containing known amounts of unlabeled 2,3,7,8-TCDD and/or other PCDD/PCDF isomers. The accuracy of measurements for performance evaluation samples should be in the range of 70-130%.

9.3 Column Performance Check Solutions

9.3.1 At the beginning of each 12-h period during which samples are to be analyzed, an aliquot of the HRGC column performance check solution shall be analyzed to demonstrate adequate HRGC resolution for selected TCDD isomers.

9.4 Method Blanks

9.4.1 A minimum of one method blank is generated with each batch of samples. A method blank is generated by performing all steps detailed in the analytical procedure using all reagents, standards, equipment, apparatus, glassware, and solvents that would be used for a sample analysis, but omit addition of the adipose tissue.

9.4.1.1 The method blank must contain the same amounts of Carbon-13 labeled internal quantitation standards that are added to samples before bulk lipid cleanup.

9.4.1.2 An acceptable method blank exhibits no positive response for any of the characteristic ions monitored.

9.4.1.2.1 If the above criterion is not met, solvents, reagents, spiking solutions, apparatus, and glassware are checked to locate and eliminate the source of contamination before any samples are extracted and analyzed.

9.4.1.2.2 If new batches of reagents or solvents contain interfering contaminants, purify or discard them.

9.5 Control Samples -- Control samples are prepared from a bulk sample(s) of human adipose tissue or similar matrix (e.g., porcine fat). This material is prepared by blending the tissue with methylene chloride, drying the extract by eluting through anhydrous sodium sulfate, and removing the methylene chloride using rotoevaporation at elevated temperatures (80°C). The evaporation process should be extended to ensure all traces of the extraction solvent have been removed. The resulting oily matrix (lipid) is subdivided into 10-g aliquots which are analyzed with each sample batch. The results of the individual analysis will be used to give a measure of precision from batch to batch over an entire program. Sufficient tissue should be extracted to provide a homogeneous lipid matrix that can be used over the total analysis program. Enough lipid matrix is necessary to prepare the spiked samples describe in Section 9.6.

9.6 Spiked Samples -- Spiked lipid samples are prepared using a portion of the homogenized lipid described in Section 9.5. Sufficient spiked lipid matrix is prepared to provide a minimum of one spiked sample per sample batch. It is recommended that a minimum of three spiked levels of the matrix are prepared ranging from 10 to 50 times the estimated limit of detection for each compound. Each analysis of spiked sample must be accompanied by analysis of a control sample in order to make the necessary corrections for background contribution before determining the accuracy of the method (Equation 9-1).

$$\text{Accuracy (\%)} = 100\% \times \frac{\text{Conc. spiked sample-conc. control sample}}{\text{Spike level}} \qquad \text{Eq. 9-1}$$

9.7 Duplicate Sample Analysis -- When possible a duplicate analysis of specific samples is included in the sample batch as an additional measure of method precision. It is suggested that the total tissue sample is extracted to isolate lipids material and then subdivided for duplicate analysis. Precision is calculated as relative percent difference (RPD) where the differences in the duplicate measurements (for each analyte) is divided by the average of the two measurements and multiplied by 100%.

9.8 External Samples -- Samples submitted as blinds to the analyst may consist of either performance solutions of PCDD and PCDF congeners or spiked sample matrices. These performance solutions or samples should be submitted by a source external to the analytical program (QA unit of analysis laboratory or independent laboratory). Performance audit solutions are intended to evaluate instrument calibration and quantitation procedures. Spiked blind samples must be accompanied by the corresponding unspiked samples to correct concentrations for background concentration. The blind spiked samples are intended to evaluate the total analytical procedure. The analyst must keep in mind that it is necessary to compare differences in standard sources for each type of external sample.

10. SAMPLE PRESERVATION AND HANDLING

All adipose tissue samples must be maintained at less than -20°C from time of collection. The analyst should instruct the collaborator collecting the sample(s) to avoid the use of chlorinated materials. Samples are handled using stainless steel forceps, spatulas, or scissors. Aliquots of samples removed from sample bottles not used for analysis are disposed rather than returned to the sample vial. All sample bottles (glass) are cleaned as specified in Section 6.4.10. Teflon®-lined caps should be used. As with any biological sample, the analyst should avoid any undue exposure.

11. SAMPLE EXTRACTION

11.1 Extraction of Adipose Tissue

11.1.1 Accurately weigh to the nearest 0.01 g a 10-g portion of a frozen adipose tissue sample into a culture tube (2.2 x 15 cm).

Note: Sample size may be smaller, depending on availability.

11.1.2 Addition of internal quantitation standards

Allow the adipose tissue specimen to reach room temperature and then add the carbon-13 internal quantitation spiking solution (Section 7.6) such that it delivers 500 to 2,500 pg of each of the surrogates specified in Table 4 in a 100-µL volume.

11.1.3 Add 10 mL of methylene chloride and homogenize the mixture for approximately 1 min with a Tekmar Tissuemizer®.

11.1.4 Allow the mixture to separate and decant the methylene chloride extract from the residual solid material using a disposable pipette. The methylene chloride is eluted through a filter funnel containing a plug of clean glass wool and 5 to 10 g of anhydrous sodium sulfate. The dried extract is collected in a 100-mL volumetric flask.

11.1.5 A second 10-mL aliquot of methylene chloride is added to the sample and homogenized for 1 min. The methylene chloride is decanted, dried, and transferred to the 100-mL volumetric flask as specified in Section 11.1.3

11.1.6 The culture tube is rinsed with at least two additional aliquots (10 mL each) of methylene chloride, and the entire contents are transferred to the filter funnel containing the anhydrous sodium sulfate. The filter funnel and contents are rinsed with additional methylene chloride (20 to 40 mL). The total eluent from the filter funnel is collected in the 100-mL volumetric flask. Discard the sodium sulfate.

11.1.7 The final volume of the extract for each sample is adjusted to 100 mL in the volumetric flask using methylene chloride.

11.2 Lipid Determination

11.2.1 Preweigh a clean 1-dram glass vial to the nearest 0.0001 g using an analytical balance tared to zero.

11.2.2 Accurately transfer 1.0 mL of the final extract (100 mL) from Section 11.1.7 to the 1-dram vial. Reduce the volume of methylene chloride from the extract using a water bath (50-60°C) gentle stream of purified nitrogen until an oil residue remains.

11.2.3 Accurately weigh the 1-dram vial and residue to the nearest 0.0001 g and calculate the weight of lipid present in the vial based on difference. Nitrogen blow-down is continued until a constant weight is achieved.

11.2.4 Calculate the percent lipid content of the original sample to the nearest 0.1% as shown in Equation 11-1.

$$\text{Lipid content, LC (\%)} = \frac{W_{LR} \times V_{EXT}}{W_{AT} \times V_{AL}} \times 100\% \qquad \text{Eq. 11-1}$$

where: W_{LR} = weight of the lipid residue to the nearest 0.0001 g calculated from Section 11.2.3;

V_{EXT} = total volume of the extract in mL from Section 11.1.6 (100.0 mL);

W_{AT} = weight of the original adipose tissue samples to the nearest 0.01 g from Section 11.1.1; and

V_{AL} = volume of the aliquot of the final extract in mL used for the quantitative measure of the lipid residue (1.0 mL).

11.2.5 Record the lipid residue measured in Section 11.2.3 and the percent lipid content calculated from Section 11.2.4.

11.3 Extract Concentration

11.3.1 Quantitatively transfer the remaining extract volume (99.0 mL) to a 500-mL Erlenmeyer flask. Rinse the volumetric flask with 20 to 30 mL of additional methylene chloride to ensure quantitative transfer.

11.3.2 Place the Erlenmeyer flask on a hot plate at 40°C to remove solvent until an oily residue remains.

12. CLEANUP PROCEDURES

12.1 Bulk Lipid Removal

12.1.1 Add a total of 200 mL of n-hexane to the spiked lipid residue in the 500-mL Erlenmeyer flask.

12.1.2 Slowly add, with stirring, 100 g of the 40% w/w sulfuric acid impregnated silica gel (Section 7.1.3). Stir with a magnetic stir-plate for 2 h.

12.1.3 Allow solids to settle and decant liquid through a powder funnel containing 20 g of anhydrous sodium sulfate and collect in a 500-mL sample bottle.

12.1.4 Rinse solids with two 50-mL portions of hexane. Stir each rinse for 15 min, decant, and dry by elution through sodium sulfate combining the hexane extracts from Section 12.1.3.

12.1.5 After the rinses have gone through the sodium sulfate, rinse the sodium sulfate with an additional 25 mL of hexane and combine with the hexane extracts from Section 12.1.4.

12.1.6 Prepare an acidic silica column as follows: Pack a 1 cm x 10 cm chromatographic column with a glass wool plug, add approximately 25 mL of hexane, add 1.0 g of silica gel (Section 7.1.2) and allow to settle, then add 4.0 g of 40% w/w sulfuric acid impregnated silica gel (Section 7.1.3) and allow to settle. Pack a second chromatographic column (1 cm x 30 cm) with a glass wool plug, add approximately 25 mL of hexane, add 6.0 g of acidic alumina (Section 7.1.1), and allow to settle and then top with a 1-cm layer of sodium sulfate (Section 7.2). Elute the excess hexane solvent through the columns until the solvent level reaches the top of the chromatographic packing. Inspect columns to ensure they are free of channels and air bubbles. Wash the alumina column with 40 mL of 50% v/v methylene chloride/hexane. Remove the methylene chloride from the adsorbent by eluting the column with an additional 100 mL of hexane. Elute the excess solvent from the column until the solvent level reaches the top of the sodium sulfate layer.

12.1.7 Quantitatively transfer the hexane extract from the Erlenmeyer flask (Sections 12.1.3 through 12.1.5) to the silica gel column reservoir. Allow the hexane extract to percolate through the column and collect in a KD concentrator.

12.1.8 Complete the elution of the extract from the silica gel column with 50 mL of hexane in the KD concentrator. Concentrate the eluate to approximately 1.0 mL, using nitrogen blow-down as necessary.

Note: If the 40% sulfuric acid/silica gel is noted to be highly discolored throughout the length of the adsorbent bed it is necessary to repeat the cleaning procedure beginning with Section 12.1.1.

12.2 Separation of Chemical Interferences

12.2.1 Transfer the concentrate (1.0 mL) to the top of the alumina column. Rinse the K-D assembly with two 1.0-mL portions of hexane and transfer the rinses to the top of the alumina column. Elute the alumina column with 18 mL of hexane until the hexane level is just below the top of the sodium sulfate. Discard the eluate. Columns must not be allowed to reach dryness (i.e., a solvent "head" must be maintained).

12.2.2 Place 30 mL of 20% (v/v) methylene chloride in hexane on top of the alumina and elute the TCDDs from the column. Collect this fraction in a 50-mL culture tube.

12.2.3 Prepare an 18% Carbopak C/Celite 545® mixture by thoroughly mixing 3.6 g of Carbopak C (80/100 mesh) and 16.4 g of Celite 545® in a 40-mL vial. Activate at 130°C for 6 h. Store in a desiccator. Cut off a clean 5-mL disposable glass pipet (6 to 7 mm ID) at the 4-mL mark. Insert a plug of glass wool and push to the 2-mL mark. Add 500 mg of the activated Carbopak/Celite mixture followed by another glass wool plug. Using two glass rods, push both glass wool plugs simultaneously towards the Carbopak/Celite mixture and gently compress the Carbopak/Celite plug to a length of 3 to 3.5 cm. Pre-elute the column with 2 mL of toluene followed by 1 mL of 75:20:5 methylene chloride/methanol/ benzene, 1 mL of 1:1 cyclohexane in methylene chloride, and 2 mL of hexane. The flow rate should be less than 0.5 mL/min. While the column is still wet with hexane, add the entire eluate (30 mL) from the alumina column (Section 12.2.2) to the top of the column. Rinse the culture tube which contained the extract twice with 1 mL of hexane and add the rinsates to the top of the column. Elute the column sequentially with two 1-mL aliquots of hexane, 1 mL of 1:1 cyclohexane in methylene chloride, and 1 mL of 75:20:5 methylene chloride/methanol/benzene. Turn the column upside down and elute the PCDD/PCDF fraction with 20 mL of toluene into 6-dram vial.

12.2.4 Using a stream of nitrogen, reduce the toluene volume to approximately 1 mL. Carefully transfer the concentrate into a 1-mL minivial and reduce the volume to about 200 µL using a stream of nitrogen.

12.2.5 Rinse the concentrator tube with three washings using 500 µL of 1% toluene in methylene chloride. Concentrate to 200-500 µL and add 10 µL of the tridecane solution containing the internal recovery standard and store the sample in a refrigerator until HRGC/MS analysis.

12.2.6 Immediately prior to analysis, using a gentle stream of nitrogen at room temperature, remove toluene and methylene chloride. Submit sample to HRGC/MS once a stable 10 µL volume of tridecane is attained.

13. ANALYTICAL PROCEDURES

13.1 HRGC/MS Analysis for PCDD/PCDF

13.1.1 Once routine calibration criteria are met, the instrument is ready for sample analysis. Prior to the first sample, a blank injection of tridecane should be analyzed to document system cleanliness. If any evidence of system contamination is found, corrective action must be taken and another tridecane blank analyzed.

The typical daily sequence of injections is shown in Table 9 and Figure 3.

Note: Syringe Technique -- Congeners of PCDD/PCDF in the syringes used for HRGC/MS analysis can be problematic unless the syringes are properly handled between samples. The following procedure has been found to be very effective for PCDD/PCDF removal from contaminated syringes and will be used throughout these analyses.

- Rinse the syringe 10 times with isooctane.
- Fill the syringe with toluene and sonicate syringe and plunger in toluene for 5 min and repeat at least twice.
- Rinse the syringe 10 times with tridecane and pull up 1 µL of clean tridecane.
- Syringe is ready for use.

At no time should air be introduced into the HRGC column by using an air plug in the syringe. The oxygen present in the air plug will quickly degrade a nonbonded GC phase.

13.1.2 Inject a 1-µL aliquot of the extract into the GC, operated under the conditions previously used (Section 8.1) to produce acceptable results with the performance check solution.

Table 9. Typical Daily Sequence for PCDD/PCDF Analysis

1. Tune and calibrate mass scale versus perfluorokerosene (PFK).
2. Inject column performance mixture.
3. Inject concentration calibration solution 2.5 to 12.5 pg/μL (CS-7) solution.
4. Inject blank (tridecane).
5. Inject samples 1 through "N".
6. Inject concentration calibration solution 2.5 to 12.5 pg/μL (CS-7) solution or other concentration calibration solutions CS1 to CS8 to bracket observed sample concentration.

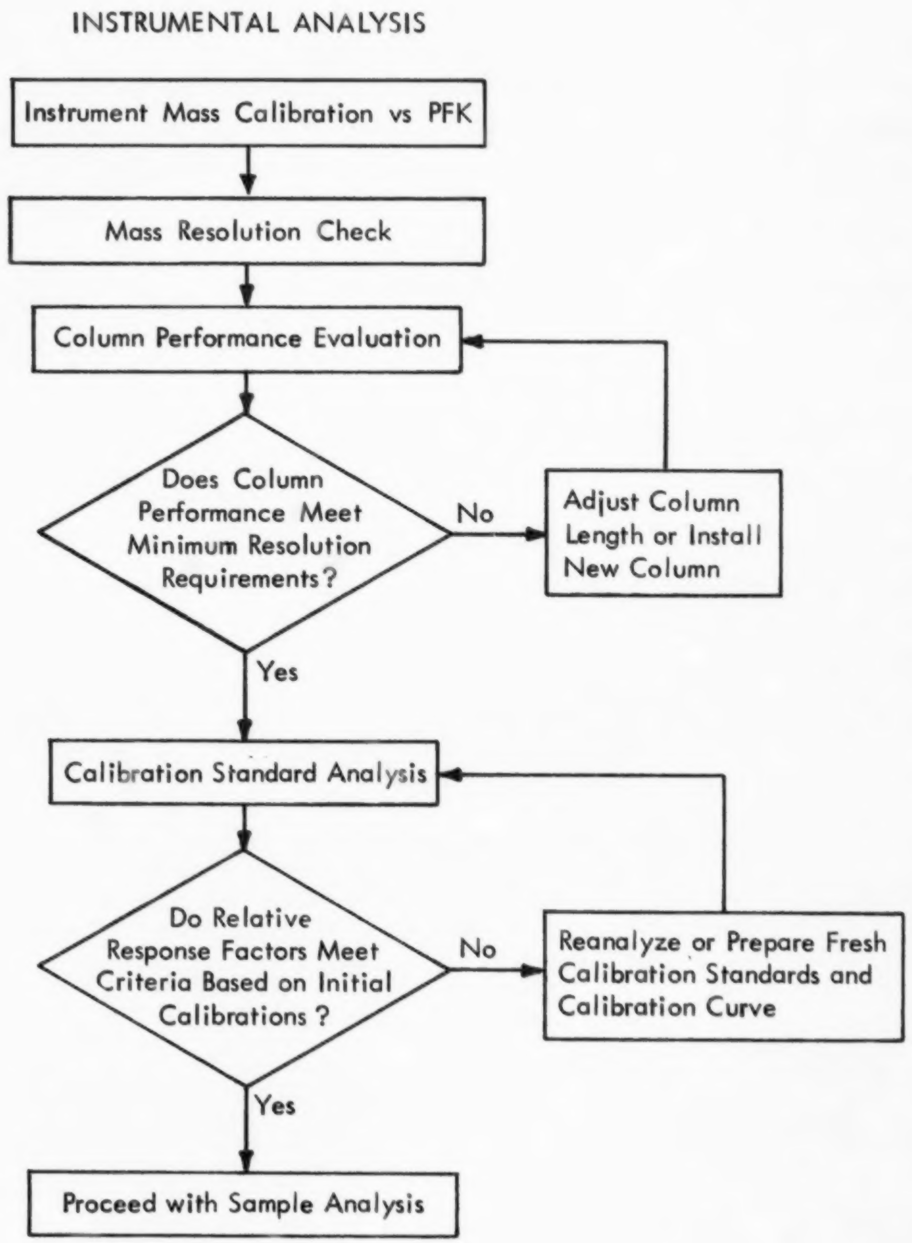

INSTRUMENTAL ANALYSIS
Instrument Mass Calibration vs PFK
Mass Resolution Check
Column Performance Evaluation
Does Column Performance Meet Minimum Resolution Requirements?
No
Adjust Column Length or Install New Column
Yes
Calibration Standard Analysis
Do Relative Response Factors Meet Criteria Based on Initial Calibrations ?
No
Reanalyze or Prepare Fresh Calibration Standards and Calibration Curve
Yes
Proceed with Sample Analysis

Figure 3. Daily QA procedures for proceeding with sample analysis.

13.1.3 Acquire SIM data according to the same acquisition and MS operating conditions previously used (Section 8.1) to determine the relative response factors.

13.1.3.1 Acquire SIM data for the characteristic ions designated in Table 6.

13.1.3.2 Instrument performance shall be monitored by examining and recording the peak areas for the recovery standard, $^{13}C_{12}$-1,2,3,4-TCDD. If this area should decrease to less than 50% of the calibration standard, sample analyses shall be stopped until the problem is found and corrected.

13.2 HRGC/HRMS Confirmation of 2,3,7,8-TCDD

The presence of 2,3,7,8-TCDD observed through the general PCDD and PCDF procedure should be confirmed using HRGC/HRMS (resolution 10,000).

13.2.1 Once the daily criteria of mass calibration, mass resolution, HRGC performance, and routine calibration are met and documented, the instrument is ready for sample analysis. Prior to the first sample, a blank injection of tridecane will be made to document system cleanliness.

The typical daily schedule for HRGC/HRMS analysis of TCDD is shown in Table 10 and Figure 3.

13.2.2 Inject a 1-μL aliquot of the extract into the GC, operated under the conditions previously used (Section 8.2) to produce acceptable results with the column performance check solution.

13.2.3 Acquire SIM data according to Section 8.2.4.3. Use the same acquisition and MS operating conditions previously used to determine the relative response factors.

13.2.3.1 Acquire SIM data for the following selected characteristic ions:

m/z	Compound
258.930	TCDD - COCl
319.897	Unlabeled TCDD
321.894	Unlabeled TCDD
331.937	$^{13}C_{12}$-2,3,7,8-TCDD, $^{13}C_{12}$-1,2,3,4-TCDD
333.934	$^{13}C_{12}$-2,3,7,8-TCDD, $^{13}C_{12}$-1,2,3,4-TCDD

Table 10. Typical Daily Schedule for HRGC/HRMS Analysis of TCDD

1. Tune and calibrate mass scale.
2. Perform mass measurement check and mass resolution check.
3. Inject column performance check solution.
4. Inject the routine concentration calibration solution (CS7) and confirm response factor consistency.
5. Inject tridecane blank.
6. Inject samples 1 through "N".
7. Inject concentration calibration solution and confirm response factor consistency.
8. Mass resolution check.

14. DATA REDUCTION

In this section, the procedures for the data reduction are outlined for the analysis of data from both the HRGC/MS method for PCDD/PCDF and the HRGC/HRMS method for 2,3,7,8-TCDD. Figure 4 presents a schematic of the qualitative criteria for identifying PCDDs and PCDFs.

14.1 Qualitative Identification

14.1.1 The ion current responses for each mass for a particular PCDD/PCDF analyte must be within ± 1 s to attain positive identification of that analyte. For example, m/z 338 and m/z 340 must have maximum peak responses that are within ± 1 s to be positively identified as a pentachlorodibenzofuran.

14.1.2 The ion current intensities for a particular PCDD/PCDF must be $\geqq 2.5$ times the noise level ($S/N \geqq 2.5$) for positive identification of that isomer.

14.1.3 The integrated ion current ratios of the analytical masses for a particular PCDD/PCDF must fall within the ranges shown in Table 7.

14.1.4 The recovery of the internal quantitation standards should be between 50 and 115%.

14.2 Quantitative Calculations

14.2.1 Relative response factors for native PCDD and PCDF analytes (RRF). RRFs are calculated from the data obtained during the analysis of concentration calibration solutions using the following formula:

$$RRF = \frac{A_{STD} \cdot C_{IS}}{A_{IS} \cdot C_{STD}} \qquad \text{Eq. 14-1}$$

where A_{STD} = the sum of the areas of the integrated ion abundances for the analyte in question. For example, for TCDD, A_{STD} would be the sum of the integrated ion abundances for m/z 320 and 322;

A_{IS} = the sum of the areas of the integrated ion abundances for the labeled PCDD/F used as the internal quantitation standard for the above analyte. For example, for $^{13}C_{12}$-2,3,7,8-TCDD, A_{IS} would be the sum of the integrated ion abundance for m/z 332 and 334.

C_{STD} = concentration of the analyte in pg/μL;

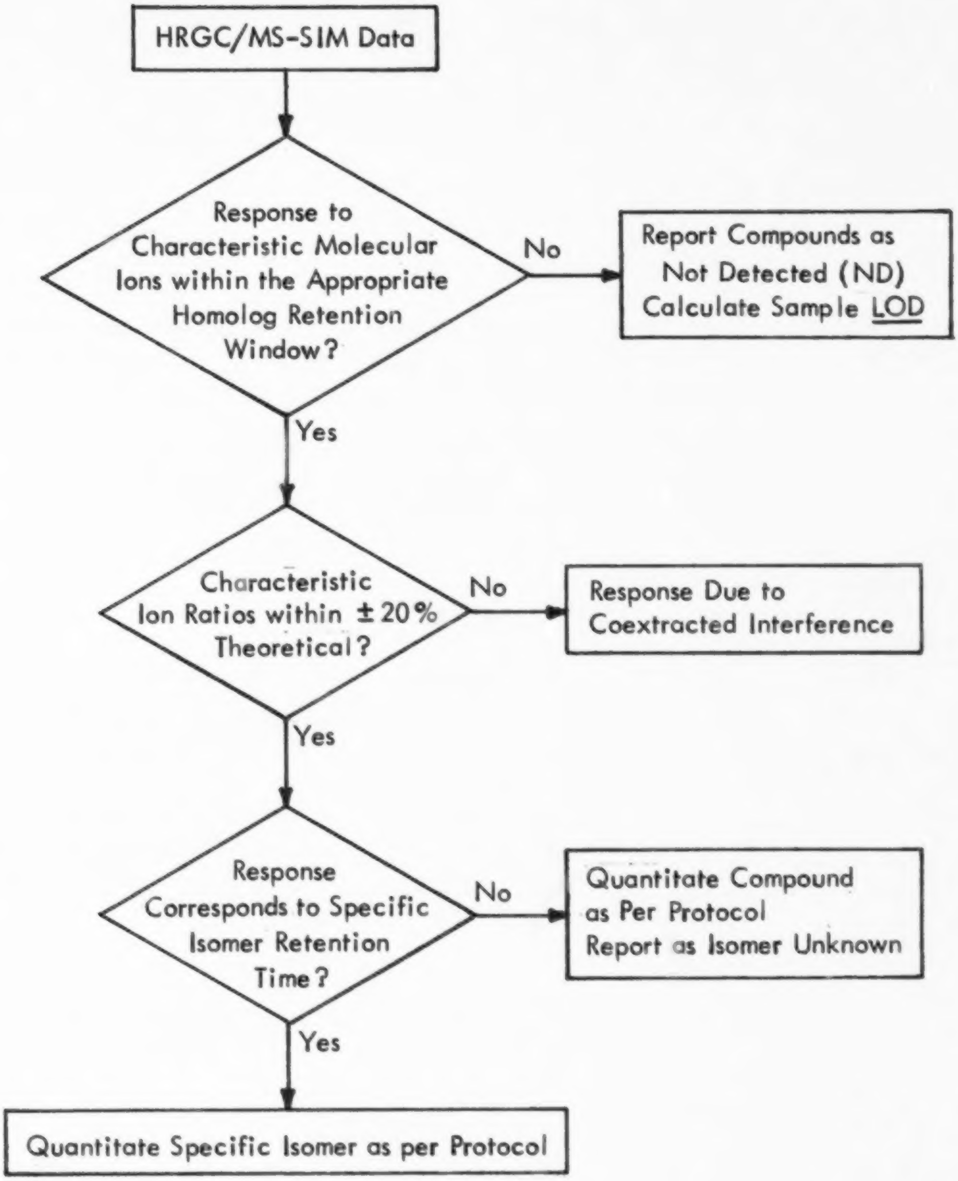

HRGC/MS-SIM Data
Response to Characteristic Molecular Ions within the Appropriate Homolog Retention Window?
No
Report Compounds as Not Detected (ND) Calculate Sample LOD
Yes
Characteristic Ion Ratios within ± 20% Theoretical?
No
Response Due to Coextracted Interference
Yes
Response Corresponds to Specific Isomer Retention Time?
No
Quantitate Compound as Per Protocol Report as Isomer Unknown
Yes
Quantitate Specific Isomer as per Protocol

Figure 4. Qualitative criteria for identifying PCDDs and PCDFs.

C_{IS} = concentration of the internal quantitation standard in pg/μL; and

Table 11 provides the pairing of target analytes to internal quantitation standards for determining RRF values for PCDD and PCDF compounds.

14.2.2 Relative response factors for the internal quantitation standards (RRF_{IS}). The RRF_{IS} values are calculated from data obtained during the analysis of concentration calibration solutions using the following formula.

$$RRF_{IS} = \frac{A_{IS} \times C_{RS}}{A_{RS} \times C_{IS}} \qquad \text{Eq. 14-2}$$

where A_{IS} and C_{IS} are defined as given in Section 14.2.1 and

C_{RS} = concentrations of the internal recovery standard in pg/μL; and

A_{RS} = the sum of the areas of the integrated ion abundances for the labeled PCDD ($^{13}C_{12}$-1,2,3,4-TCDD or $^{13}C_{12}$-1,2,3,7,8,9-HxCDD). For example, for $^{13}C_{12}$-1,2,3,4-TCDD, A_{RS} would be the sum of the integrated ion abundance for m/z 332 and 334.

Refer to Table 11 for pairing of the internal quantitation standards with the appropriate internal recovery standard.

14.2.3 Concentrations of sample components. Figure 5 presents a schematic for quantitation of PCDDs and PCDFs which meet the criteria specified in Section 14.1. Calculate the concentration of PCDD/Fs in sample extracts using the formula:

$$C_{sample} = \frac{A_{sample} \cdot Q_{IS} \cdot 100}{A_{IS} \cdot RRF \cdot W_{AT} \cdot LC} \qquad \text{Eq. 14-3}$$

where C_{sample} = the lipid adjusted concentration of PCDD or PCDF congener in pg/g;

A_{sample} = sum of the integrated ion abundances determined for the PCDD/PCDF in question;

A_{IS} = sum of the integrated ion abundances determined for the labeled PCDD/F used as the internal quantitation standard for the above analyte;

Table 11. Target Analyte/Internal Quantitation Standard and Internal Quantitation Standard/Internal Recovery Standard Pairs

Target analyte	Internal standards	
	Quantitation	Recovery
2,3,7,8-TCDD	$^{13}C_{12}$-2,3,7,8-TCDD	$^{13}C_{12}$-1,2,3,4-TCDD
2,3,7,8-TCDF	$^{13}C_{12}$-2,3,7,8-TCDF	$^{13}C_{12}$-1,2,3,4-TCDD
1,2,3,7,8-PeCDF	$^{13}C_{12}$-1,2,3,7,8-PeCDF	$^{13}C_{12}$-1,2,3,4-TCDD
2,3,4,7,8-PeCDF	$^{13}C_{12}$-1,2,3,7,8-PeCDF	$^{13}C_{12}$-1,2,3,4-TCDD
1,2,3,7,8-PeCDD	$^{13}C_{12}$-1,2,3,7,8-PeCDD	$^{13}C_{12}$-1,2,3,4-TCDD
1,2,3,4,7,8-HxCDF	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,6,7,8-HxCDF	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
2,3,4,6,7,8-HxCDF	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,7,8,9-HxCDF	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,4,7,8-HxCDD	$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,6,7,8-HxCDD	$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,7,8,9-HxCDD	$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,4,6,7,8-HpCDF	$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,4,7,8,9-HpCDF	$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
1,2,3,4,6,7,8-HpCDD	$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
OCDF	$^{13}C_{12}$-OCDD	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD
OCDD	$^{13}C_{12}$-OCDD	$^{13}C_{12}$-1,2,3,7,8,9-HxCDD

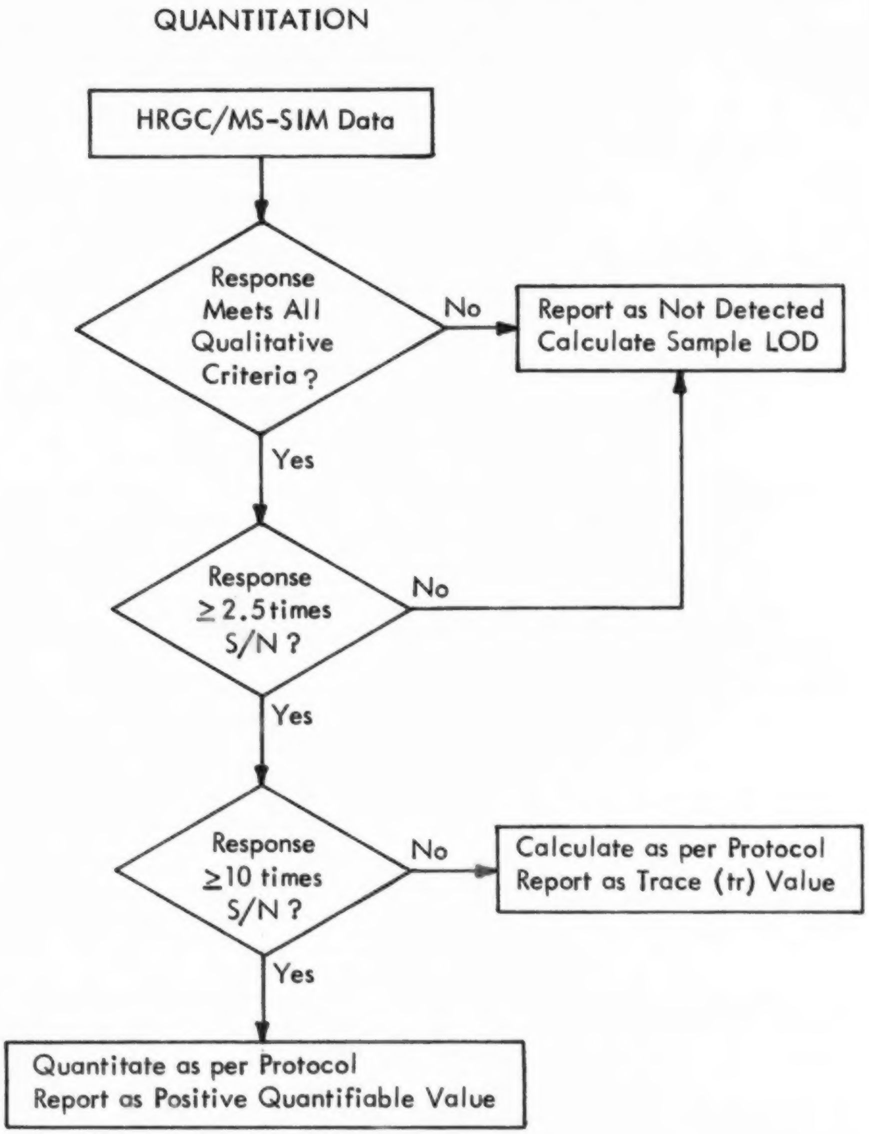

QUANTITATION
HRGC/MS-SIM Data
Response Meets All Qualitative Criteria ?
No
Report as Not Detected Calculate Sample LOD
Yes
Response ≥ 2.5 times S/N ?
No
Yes
Response ≥ 10 times S/N ?
No
Calculate as per Protocol Report as Trace (tr) Value
Yes
Quantitate as per Protocol Report as Positive Quantifiable Value

Figure 5. Procedure for quantitation of PCDDs and PCDFs in human adipose tissue.

Q_{IS} = the amount (total pg) of the labeled internal quantitation standard added to the sample prior to extraction;

RRF = relative response factor of the above analyte relative to its labeled internal quantitation standard determined from the initial triplicate calibration;

W_{AT} = weight (g) of original adipose tissue sample; and

LC = percent extractable lipid determined from Eq. 11-1.

Refer to Table 11 for pairing of target analytes with the appropraite internal quantitation standard.

Quantitative data should be classified to indicate the intensity of the signal response. Suggested qualifiers include: not detected, ND (signal-to-noise ratio is less than 2.5); trace, TR (signal-to-noise ratio is greater than or equal to 2.5 but less than 10); and positive quantifiable, PQ (signal-to-noise ratio is greater than or equal to 10).

14.2.4 Recovery of internal quantitation standards. Calculate the recovery of the labeled internal quantitation standards measured in the final extract using the formula:

$$\text{Internal Quant. Std. Percent Recovery} = \frac{A_{IS} \cdot Q_{RS}}{A_{RS} \cdot Q_{IS} \cdot RRF} \cdot 100 \qquad \text{Eq. 14.4}$$

where A_{IS} = sum of the integrated ion abundances determined for the labeled PCDD/PCDF internal quantitation standard in question;

A_{RS} = sum of the integrated ion abundances determined for m/z 332 and m/z 334 of $^{13}C_{12}$-1,2,3,4-TCDD or m/z 390 and m/z 392 of $^{13}C_{13}$-1,2,3,7,8,9-HxCDD (recovery standards)

Q_{RS} = amount (pg) of the respective recovery standard, added to the final extract;

Q_{IS} = amount (pg) the labeled internal quantitation standard added to the sample prior to extraction; and

RRF_{IS} = relative response factor for the labeled internal quantitation standard in question relative to the internal recovery standard. This value shall be the RRF determined from the initial calibration.

Refer to Table 11 for pairing of the internal quantitation standards with the appropriate target analytes.

Note: The result of calculations as presented in Section 14.2 may be off by as much as 1% due to the fact that 1 mL of the final 100 mL volume from the extraction was used for lipid determination.

14.3 Estimated Method Detection Limit

Estimated method detection limits must be calculated in situations where (1) no response is noted for a specific congener; (2) a response is noted but ion ratios are incorrect; and (3) where a response is quantitated as a trace value.

14.3.1 For samples in which no unlabeled PCDD or PCDF is detected, calculate the estimated minimum detectable concentration. The background area is determined by integrating the ion abundances for the characteristic ions in the appropriate region and relating the product area to an estimated concentration that would produce that product area.

Use the formula:

$$C_E = \frac{2.5 \cdot A_{sample} \cdot Q_{IS}}{A_{IS} \cdot \overline{RRF} \cdot W_{AT}} \qquad \text{Eq. 14-5}$$

where C_E = estimated concentration of unlabeled PCDD or PCDF required to produce A_{sample};

A_{sample} = sum of integrated ion abundances or peak heights for the characteristic ions of the unlabeled PCDD or PCDF isomer in the same group of ≧ 5 scans used to measure A_{IS}; and

A_{IS} = sum of integrated ion abundances for the appropriate ions characteristic of the respective internal quantitation standard.

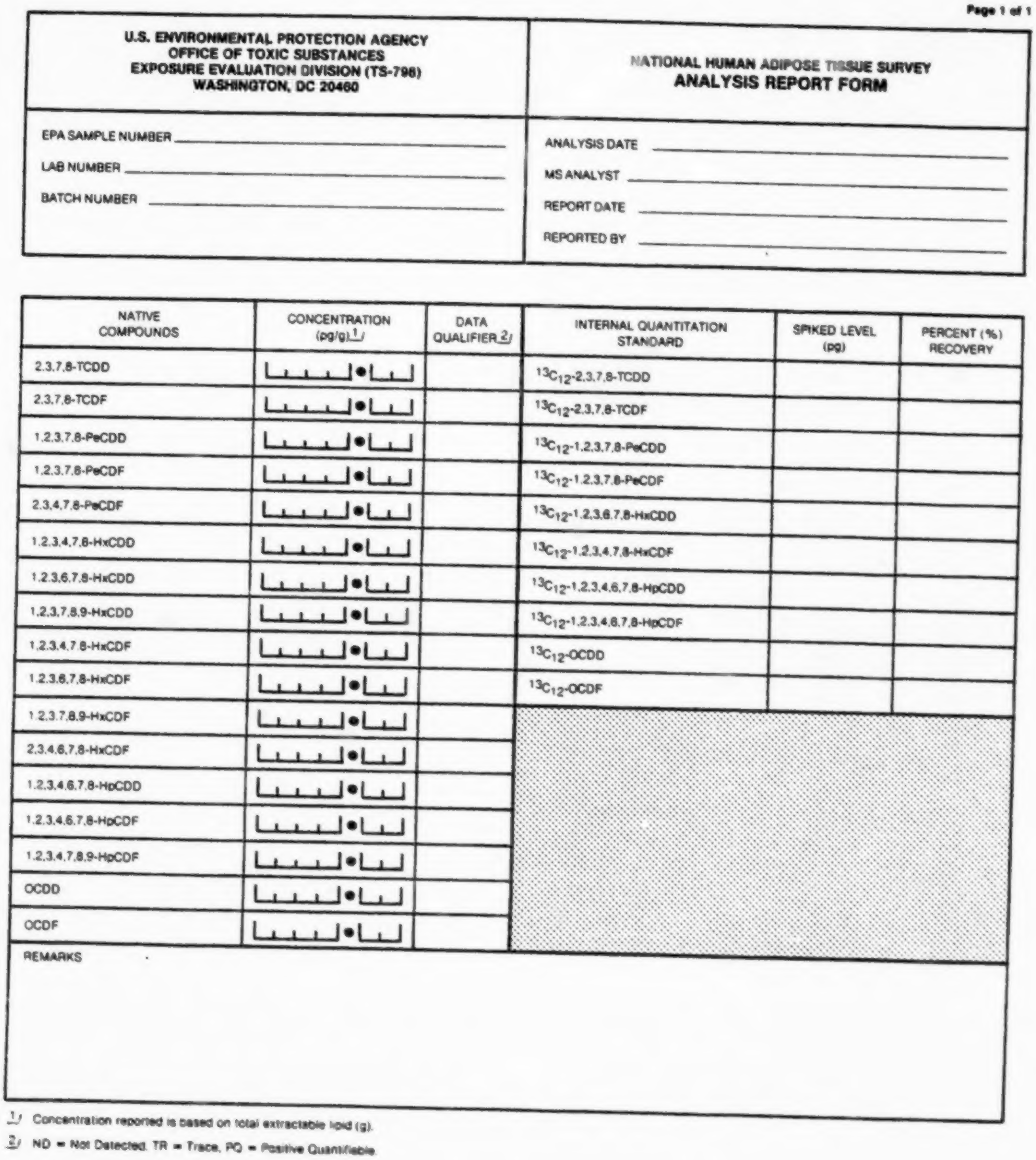
Q_{IS}, $\overline{RRF}$, and W_{AT} retain the definitions previously stated in Section 14.2. Alternatively, if peak height measurements are used for quantification, measure the estimated detection limit by the peak height of the noise in the 2,3,7,8-TCDD RT window.

14.3.2 For samples for which a response at the retention time of a specific PCDD or PCDF congener is noted, but the qualitative criteria for ion ratios are outside the acceptable range (Table 7), the estimated detection level is calculated as given in Eq. 14.3 except the values are qualified as not detected, ND, and the concentration is reported in parenthesis.

14.3.3 If a response for a specific PCDD or PCDF congener is qualified as a trace, TR, value (signal to noise is greater than or equal to 2.5 but less than 10) the analyst must also provide an estimated method detection limit. This is accomplished by using the observed signal to noise on either side of the response and calculating as given in Eq. 14-5.

15. REPORTING AND DOCUMENTATION

All data should be reported on an individual sample basis using the data report format shown in Figure 6. The analyst is required to maintain all raw data, calculations, and control charts in a format as to allow a complete external data review. Suggested data formats for tracing calculations are provided in Figure 7.



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U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF TOXIC SUBSTANCES
EXPOSURE EVALUATION DIVISION (TS-798)
WASHINGTON, DC 20460

NATIONAL HUMAN ADIPOSE TISSUE SURVEY
ANALYSIS REPORT FORM

EPA SAMPLE NUMBER ______
LAB NUMBER ______
BATCH NUMBER ______

ANALYSIS DATE ______
MS ANALYST ______
REPORT DATE ______
REPORTED BY ______

NATIVE COMPOUNDS	CONCENTRATION (pg/g) 1/	DATA QUALIFIER 2/	INTERNAL QUANTITATION STANDARD	SPIKED LEVEL (pg)	PERCENT (%) RECOVERY
2,3,7,8-TCDD	•		$^{13}C_{12}$-2,3,7,8-TCDD		
2,3,7,8-TCDF	•		$^{13}C_{12}$-2,3,7,8-TCDF		
1,2,3,7,8-PeCDD	•		$^{13}C_{12}$-1,2,3,7,8-PeCDD		
1,2,3,7,8-PeCDF	•		$^{13}C_{12}$-1,2,3,7,8-PeCDF		
2,3,4,7,8-PeCDF	•		$^{13}C_{12}$-1,2,3,6,7,8-HxCDD		
1,2,3,4,7,8-HxCDD	•		$^{13}C_{12}$-1,2,3,4,7,8-HxCDF		
1,2,3,6,7,8-HxCDD	•		$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD		
1,2,3,7,8,9-HxCDD	•		$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF		
1,2,3,4,7,8-HxCDF	•		$^{13}C_{12}$-OCDD		
1,2,3,6,7,8-HxCDF	•		$^{13}C_{12}$-OCDF		
1,2,3,7,8,9-HxCDF	•				
2,3,4,6,7,8-HxCDF	•				
1,2,3,4,6,7,8-HpCDD	•				
1,2,3,4,6,7,8-HpCDF	•				
1,2,3,4,7,8,9-HpCDF	•				
OCDD	•				
OCDF	•				

REMARKS

1/ Concentration reported is based on total extractable lipid (g).

2/ ND = Not Detected, TR = Trace, PQ = Positive Quantifiable.

Figure 6. Analysis report form.

RAW DATA SUMMARY FOR DETERMINATION OF 1,2,3,7,8-PeCDD IN HUMAN ADIPOSE TISSUE

Sample no.	Sample weight (xx.xx g)	Extractable lipid content (xx.x %)	Analysis date	Amount $^{13}C_{12}$-PeCDD (pg)	$^{13}C_{12}$-PeCDD m/z 332	$^{13}C_{12}$-PeCDD m/z 334	Ion ratio 366/368	1,2,3,7,8-PeCDD m/z 354	1,2,3,7,8 m/z 356	Ion ratio 354/356	1,2,3,7,8-PeCDD conc.[a] (pg/g)

---

[a] Value reported as concentration in extractable lipid.

Figure 7. Example of raw data summary format for the determination of 1,2,3,7,8-PeCDD in human adipose tissue.